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Cytotoxic effects of cannabidiol on tumor cells versus normal cells

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Contents

List of abbreviations

Apaf-1 ..... *Apoptosis protease-activating factor 1*
APC ..... *Adenomatous Polyposis Coli*
APC (FACS) ..... *Allophycocyanin*
APCs ..... *Antigen presenting cells*
APS ..... *Ammonium persulfate*
ATP ..... *Adenosin triphosphate*
Bax ..... *Bcl-associated X protein*
BCA ..... *Bicinchoninic Acid*
Bcl ..... *B-cell lymphoma*
BRAF ..... *v-raf murine sarcoma viral oncogene homolog B1*
cAMP ..... *Cyclic adenosine monophosphate*
CB ..... *Cannabinoid receptor*
CBD ..... *Cannabidiol*
CBG ..... *Cannabigerol*
CBN ..... *Cannabinol*
CD45 ..... *Lymphocyte common antigen*
CEA ..... *Carcinoembryonic antigen*
CFA ..... *Colony formation assay*
CNS ..... *Central nervous system*
CRC ..... *Colorectal carcinoma*
DMEM ..... *Dulbecco's modified eagle's medium*
DMSO ..... *Dimethyl sulfoxide*
DNA ..... *Deoxyribonucleic acid*
EDTA ..... *Ethylenediamine tetraacetic acid*
ER ..... *Endoplasmic reticulum*
ERK ..... *Extracellular signal-regulated kinase*
FACS ..... *Fluorescence-Activated Cell Sorting*
FCS ..... *Fetal calf serum*
FITC ..... *Fluorescein isothiocyanate*
FSC ..... *Forward Scatter*
GPR55 ..... *G protein-coupled receptor 55*

HNPCC ..... *Hereditary nonpolyposis colorectal cancer*
HSP ..... *Heat shock protein*
ICAM-1 ..... *Intercellular adhesion molecule 1*
Id ..... *Inhibitor of DNA binding*
IFN ..... *Interferon*
Ig ..... *Immunoglobulin*
IL ..... *Interleukin*
KRAS ..... *Kirsten Rat Sarcoma*
LAK ..... *Lymphokine-activated killer*
LDH -/- ..... *Lactate dehydrogenase A and B knockout*
LDHA ..... *Lactate dehydrogenase A*
LDHB ..... *Lactate dehydrogenase B*
LSM ..... *Lymphocyte separation medium*
MAPK ..... *Mitogen activated kinase*
MHC ..... *Major histocompatibility complex*
MS ..... *Multiple sclerosis*
mTOR ..... *Mammalian target of rapamycin kinase*
NK ..... *Natural killer cell*
PBMC ..... *Peripheral blood mononuclear cells*
PBS ..... *Phosphate-buffered saline*
PE ..... *Plating efficiency*
PI ..... *Propidium Iodide*
RIPA ..... *Radioimmunoprecipitation assay*
ROS ..... *Reactive oxygen species*
rpm ..... *Revolutions per minute*
RPMI ..... *Roswell-Park Memorial Institute*
SDS ..... *Sodium dodecyl sulfate*
SF ..... *Survival fraction*
SSC ..... *Side Scatter*
TBST ..... *Tris-buffered saline with Tween20*
TEMED ..... *Tetramethylethylenediamine*
THC ..... *Tetrahydrocannabinol*
TNF-α ..... *Tumor necrosis factor α*
TRIS ..... *Tris(hydroxymethyl)aminomethane*

TRPV1 ..... *Transient receptor potential cation channel subfamily V member 1*

TRPV2 ..... *Transient receptor potential cation channel subfamily V member 2*

TRPV4 ..... *Transient receptor potential cation channel subfamily V member 4*

VR1 ..... *Vanilloid receptor type 1*

WNT ..... *Wingless-related integration site*

WT ..... *Wild type*

1 Introduction

1.1. Colorectal Cancer

1.1.1. Epidemiology

Colorectal cancer (CRC) belongs to the most commonly diagnosed cancers. Observing a modern and progressive country like the US, the lifetime probability of being diagnosed with invasive cancer lies around 39 %. Both in estimated cases and estimated deaths, CRC takes the third place in the statistics for men and women (Siegel, Miller, Fuchs, & Jemal, 2021; Siegel, Miller, & Jemal, 2020). CRC is a disease of the elderly, the average age at the time of the diagnosis being 66 (Fearon, 2011). The five-year survival rate is extremely dependent on the stage of the disease, all stages combined being 64 % considering the years 2009 to 2015 in the US (Siegel et al., 2020).

To enable the early detection of the cancer, a regular screening from the age of 45 is recommended. Colonoscopies and stool-based tests are the most common screening methods, allowing to detect occult blood and transformations in the colonic mucosa (R. A. Smith et al., 2018). The probability of a malignant transition of the colon cells can increase with a variety of risk factors, including alcohol, smoking, diabetes, high meat intake and obesity (Huxley et al., 2009; O'Sullivan et al., 2022).

1.1.2. Histology and Pathogenesis

Fearon and Vogelstein proposed a model for the development of the CRC in 1990. They described that a step by step accumulation of genetic alterations results in a colorectal neoplasia, later to be called the adenoma-carcinoma sequence. Healthy tissue develops to an adenomatous polyp or so called adenoma, which transits to the malignant carcinoma (Fearon & Vogelstein, 1990). Supporting this thesis, regions of adenomatous epithelium can remain in colorectal carcinoma lesions and beginning foci of neoplastic cells can be observed in adenomas (Fearon, 2011). However, only a small part of adenomas progress to carcinomas and the process can take years to decades (Stryker et al., 1987). The critical characteristics of adenomas are an

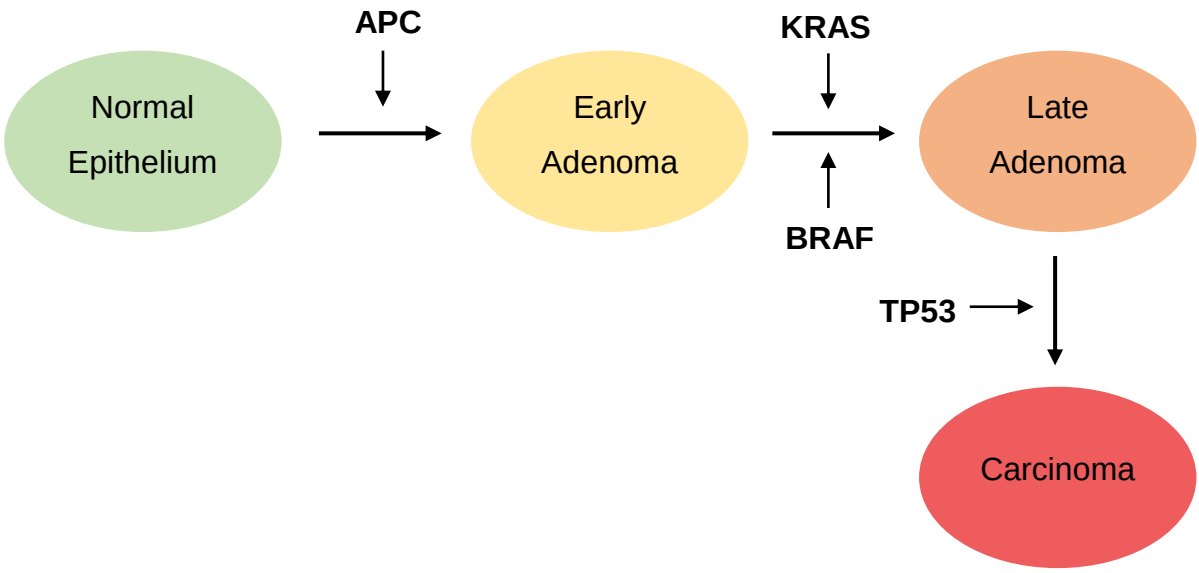

Figure 1.1: Basic scheme for the polyp-to-carcinoma progression

increasing size and villous growth patterns, both indicating a higher risk for dysplasia (Herold, 2016; Myers & Arora, 2022).

A distinction is made between sporadic and hereditary CRC, having partially different pathways of pathogenesis. The APC/β-Catenin/WNT signaling pathway is the most common pathway to be mutated in CRCs. 70-80 % of sporadic CRCs have a somatic mutation of the tumor suppressor Adenomatous Polyposis Coli (APC) leading to an abnormal activation of the WNT signaling and thereby initiating the adenoma-carcinoma sequence ("Comprehensive molecular characterization of human colon and rectal cancer," 2012; Fearon, 2011). Approximately 40 % of CRCs show activating mutations in the proto-oncogene KRAS. KRAS activates the mitogen activated kinase (MAPK) pathway through BRAF, which supports cell growth and survival. In the adenoma-carcinoma progression, KRAS mutations occur after APC mutations in the early adenoma stage. The BRAF gene is also mutated relatively often with 10-15 % of the cases. Lastly, in 50 % of the CRCs, the transformation from late adenoma stage to frank malignancy is enabled through the mutation of the most famous tumor suppressor gene: TP53. This gene plays a major part in the regulation of the cell cycle progression and apoptosis (Chung, 2000; Grady & Pritchard, 2014; La Vecchia & Sebastián, 2020). The critical genes of the typical pathway involved in the tumorigenesis are illustrated in Figure 1.1, showing that only an accumulation of different mutations leads to a malignant transition.

Up to a third of the CRC cases may be explained through inheritance, as twin studies indicate (Lichtenstein et al., 2000). Still, only 5-10 % of the diagnosed CRCs can be explained through defined hereditary cancer syndromes (Xue et al., 2018). The most researched forms are hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome, accounting for around 3-4 % of the CRC cases and familial adenomatous polyposis (FAP), responsible for nearly 1 % of the CRC cases. Germ line mutations in critical genes play a pivotal role in the early and partially very aggressive progression of these diseases (Rustgi, 2007). Most patients affected with the autosomal-dominant disease FAP show a germ line mutation in the APC gene, leading to the development of hundreds of colorectal adenomas (Nishisho et al., 1991; Weitz et al., 2005). HNPCC also presents through an autosomal-dominant inheritance pattern, however, with a different pathway of tumorigenesis. A germ line mutation of mismatch repair (MMR) genes causes an inactivation of the system that recognizes base pair mismatches in the DNA during the replication process. Out of those genes encoding for the repairing proteins, MLH1 and MSH2 are the most frequent to be inactivated. The clinical picture of HNPCC is not only characterized by an early-onset of colon cancer, but also by several extracolonic tumors, such as of the genitourinary system, biliary system, CNS and small bowel (Grady, 2003; Ma et al., 2018).

1.1.3. Diagnostics

As mentioned above, effective screening programs from the age of 45 are offered to enable a diagnosis at an early stage. The fecal occult blood test allows the identification of very small amounts of blood in the feces, which can occur through a bleeding neoplastic lesion in the colon. A colonoscopy can not only detect both polyps and cancerous lesions but also remove the transformed mucosa, preventing the polyps to progress into carcinomas. The procedure allows a safe way to provide a histological diagnosis (Hawk & Levin, 2005; Kanth & Inadomi, 2021).

Further diagnostics should always be realized if the histological analysis indicates a transformed epithelium or the clinical aspects of colonic cancer are noticed. These unspecific signs include diarrhea and/or constipation, abdominal pain, a palpable abdominal mass, rectal bleeding, iron deficiency anemia, loss of weight and deterioration of the general condition (Lehnert, Schaible, & Herfarth, 1999; Ridereau-Zins, 2014).

Since a quarter of the CRC cases present with distant metastases at the time of the initial diagnosis, preoperative imaging is crucial. The sonography plays a particular role in finding metastases in the liver, whereas pulmonal metastases are detected through chest X-ray (Herold, 2016; Schmiegel et al., 2017). Staging in rectal carcinoma additionally includes a rectoscopy in order to detect the height of the tumor and an MRI for local staging (Vogl et al., 2019).

Depending on the stage of the disease, around a third of the patients show elevated levels of the tumor marker CEA in the serum. However, this marker is not tumor specific and only relevant in the follow-up care, where it indicates a relapse (Schmiegel et al., 2017).

1.1.4. Therapy

The curative surgical treatment stipulates an open or laparoscopic en-bloc-resection of the cancer bearing colon or rectum section along with its corresponding lymph drainage region. Depending on the localization and mass, distant metastases might also be surgically removed (Schmiegel et al., 2017).

When treating colon cancer, an adjuvant chemotherapy should only be realized if the requirement of an R0 resection of the primary tumor is fulfilled, starting within 8 weeks after the operation. However, this procedure is only recommended in UICC stage III and possible in stage II, but not required in stage I (Figueredo et al., 1997; Merkel et al., 2016). The standard chemotherapy protocol of stage III patients consists of the drugs folinic acid, 5-fluorouracil and oxaliplatin, naming the therapy FOLFOX (T. Andre et al., 2004; Gelibter et al., 2019). Since oxaliplatin failed to show a benefit in clinical trials of stage II patients, 5-fluoruracil is used as a monotherapy in these cases (Tournigand et al., 2012). The very effective first-line chemotherapy plan FOLFOXIRI adds the topoisomerase inhibitor irinotecan to the scheme, increasing the side effects to a level that only patients in a good general condition can benefit from the combination (Cremolini et al., 2015; Vogl et al., 2019). The anti-EGFR antagonist cetuximab seems to optimize the impact of the first-line chemotherapy in KRAS wildtype patients (Biller & Schrag, 2021; Heinemann et al., 2014).

When treating rectal cancer, different procedures are performed depending on the tumor height. Patients with rectal carcinoma of UICC stage II and III situated in the lower or middle third of the rectum should receive a neoadjuvant radio-chemotherapy (Sauer et al., 2012; Vogl et al., 2019) and the regime should include capecitabine or

5-fluoruracil (Hofheinz et al., 2012). If located on the upper third and low risk constellation, the approach is similar as in colon cancer with an adjuvant chemotherapy (Folkesson et al., 2005; Peeters et al., 2007). Total mesorectal excision has become the standard surgical procedure for rectal cancer, resulting in increased survival and reduced local recurrence (Park et al., 2018).

Regular follow-ups are recommended after R0 resection in UICC stage II and III (Secco et al., 2002; Vogl et al., 2019). This involves the determination of the tumor marker CEA every six months for at least two years, an elevating level indicating further diagnostics (Duffy et al., 2003; Haria et al., 2021). A complete colonoscopy should be performed within six months after the surgery and repeated one year after, from then on, every five years if the diagnostic findings were inconspicuous (Rulyak, Lieberman, Wagner, & Mandelson, 2007; T. Wang et al., 2009). In order to detect lung metastases, a chest X-ray can be performed annually in patients with stage II and III rectal cancer up to the fifth year (Vogl et al., 2019). Other than that, physicians should advise their patients to get physically active to decrease their risk of relapse and improve their survival (Meyerhardt, Giovannucci, et al., 2006; Meyerhardt, Heseltine, et al., 2006).

In an UICC stage IV case, the aim of the therapy always depends on the general condition of the patient. Generally, the therapeutic approach in the metastatic stage of colorectal cancer can still be curative, unlike many other cancers (Giuliante et al., 2021). If the risk of suffering of extreme side effects after surgical or cytostatic treatments is too high, a palliative care plan is advisable. The World Health Organization defines palliative care as follows: “Palliative care is an approach that improves the quality of life of patients and their families facing the problems associated with life-threatening illness, through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other problems, physical, psychosocial and spiritual.” (Sepulveda, Marlin, Yoshida, & Ullrich, 2002). Taken together, in a very advanced stage, an individual procedure is advisable, and the focus should lie on improving quality of life rather than increasing survival.

1.2. Cannabidiol (CBD)

1.2.1. Overview

Cannabinoids belong to the species *Cannabis sativa* L., a hemp plant from Central Asia, which has been used in various fields since ancient times (C. M. Andre, Hausman, & Guerriero, 2016; Russo et al., 2008). Cannabinoids represent the most studied secondary metabolite of hemp. Around 100 different cannabinoids have been characterized so far (Appendino, Chianese, & Taglialatela-Scafati, 2011), the psychoactive Δ^9-tetrahydrocannabinol (THC) portraying the most famous agent of them. Next to THC, cannabidiol (CBD), cannabinol (CBN) and cannabigerol (CBG) are other well-known representatives of the group of phytocannabinoids (Pisanti et al., 2017). After THC, CBD is the second main component of *C. sativa*, accounting for up to 40 % of the plant (Campos, Moreira, Gomes, Del Bel, & Guimaraes, 2012). CBD first got isolated in 1940 (Adams, Hunt, & Clark, 1940) and its exact structure got revealed in the 1960ies (Mechoulam & Shvo, 1963).

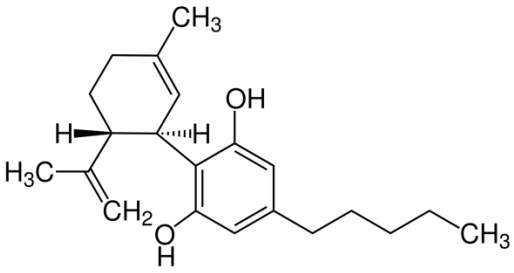

Figure 1.2: Structure of CBD

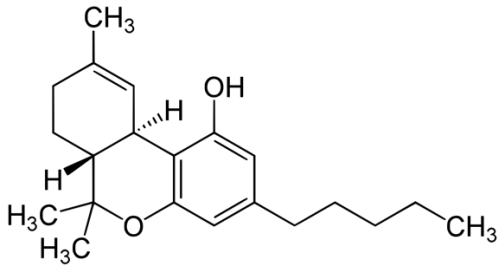

Figure 1.3: Structure of THC

Although the structure of CBD seems very similar to THC, the pharmacological profile still shows drastic differences (Burstein, 2015). The non-psychoactive drug CBD shows only low affinity to the receptors CB_1 and CB_2 compared to other cannabinoids (Bisogno et al., 2001; Showalter, Compton, Martin, & Abood, 1996). Since the receptor CB_1 is mainly located in the brain, it results in the lack of psychoactivity of CBD in contrast to THC (Galiegue et al., 1995). Instead, CBD has been observed to promote its activity through various other receptors. It causes an activation of the vanilloid receptor type 1 (VR1) and thereby induces an increase in cytosolic Ca^{2+} concentration like the VR1 agonist capsaicin (Bisogno et al., 2001). Additionally, CBD acts as an agonist for the human 5-HT1A receptor, promoting an anxiolytic effect (Gomes,

Resstel, & Guimaraes, 2011). Just like the known agonist serotonin, CBD can decrease cAMP concentration through a G protein dependent receptor system (Russo, Burnett, Hall, & Parker, 2005). Furthermore, an inhibitory effect of CBD on the GPR55 receptor has been observed, preventing cells of the innate immune system to increase the production of pro-inflammatory agents such as IL-12, IFN-γ and TNF-α (Chiurchiu, Lanuti, De Bardi, Battistini, & Maccarrone, 2015).

1.2.2. Therapeutic Relevance

Since CBD acts as a target for multiple receptors located in numerous organ systems, many effects have been observed so far. In the following, CBD is elaborated on with regard to its relevance as a therapeutic agent.

CBD appears to be neuroprotective, showing its anti-inflammatory (Walter & Stella, 2004) and anti-apoptotic (Iuvone et al., 2004) effects in the neurodegenerative disease Alzheimer's. Both *in vitro* and *in vivo*, the β-amyloid induced microglial activation - a known mechanism of the disease - is prevented by CBD (Martin-Moreno et al., 2011). It also shows neuroprotective impacts in the treatment of Parkinson's disease (Patricio, Morales-Andrade, Patricio-Martínez, & Limón, 2020), Huntington's disease (Sagredo et al., 2011) and other neurological diseases such as multiple sclerosis (MS) by its anti-inflammatory influence (Kozela et al., 2011). But also inflammation in other organ systems apart from the CNS may be treated with CBD, such as rheumatoid arthritis (Jelínek et al., 2022) and inflammatory bowel disease. Mechanisms to contain the inflammation by CBD are amongst others the reduction of TNF-α expression, the inhibition of macrophage recruitment and reduction of IL-6/IL-8 production of rheumatoid arthritis synovial fibroblasts (De Filippis et al., 2011; Lowin et al., 2020). The first ever clinical trial in patients with Crohn's disease also showed beneficial effects of CBD in the treatment (Naftali, Lev, Yablecovitch, Half, & Konikoff, 2011). As mentioned above, anxiolytic effects promoted through the 5-HT1A receptor have been observed as well, making it a possible therapeutic agent in the treatment of social anxiety (de Mello Schier et al., 2014). Even the cardiovascular system seems to profit from CBD, as it reduces the myocardial inflammation and shows anti-oxidant properties, suggesting CBD to be a possible drug in the treatment of ischemia-reperfusion injury (Durst et al., 2007; Kicman & Toczek, 2020). Also, antibacterial

effects have been demonstrated with cannabinoids, showing potent activity against methicillin-resistant Staphylococcus aureus (Appendino et al., 2008).

Discussing CBD as a licensed drug on the current market, it is part of the drug Sativex®, which is an approved medication for treatment of therapy-resistant spasticity in MS. Sativex® is a sublingual spray and consists of equal doses of THC and CBD, existing on the German market since 2011 (Grotenhermen & Müller-Vahl, 2012). Since 2017, any medical practitioner in Germany is allowed to prescribe cannabis flowers and extracts to their patients. In the law, indications were explicitly left out, as not all effects of the drug are fully understood to date. Still, the range of application seems to be high. Common indications are neuropathic pain, loss of appetite and nausea, but since cannabis also shows benefits in studies concerning other complaints, indications like atopic dermatitis, glaucoma, arthritis, inflammatory bowel disease, depression and sleeping disorders are possible. However, most types of prescribed cannabis flowers contain only a low share of CBD compared to THC, which is the main component of most supplements (Müller-Vahl & Grotenhermen, 2017). Still, the major advantage of CBD in contrast to THC is the lack of adverse effects on the CNS. Therefore CBD poses a promising therapeutic target with a high safety profile (Iffland & Grotenhermen, 2017).

1.2.3. Role in Cancer Therapy

Cannabinoids have shown beneficial effects in the treatment of nausea and loss of appetite, which are frequent side effects of cytostatic drugs used in cancer therapy (Fraguas-Sánchez & Torres-Suárez, 2018). However, not only adverse effects can be reduced, but also direct antitumor activity has been observed. In several studies, *in vivo* and *in vitro*, CBD-mediated antineoplastic activity in human breast carcinoma cells was detected (Fraguas-Sánchez, Fernández-Carballido, Simancas-Herbada, Martin-Sabroso, & Torres-Suárez, 2020; McAllister et al., 2011; Shrivastava, Kuzontkoski, Groopman, & Prasad, 2011). Various pathways seem to be influenced by CBD. The Id-1 protein, which is associated with a proliferative and invasive character, has been demonstrated to show a downregulation in its gene expression after CBD treatment. This happens both directly through inhibition of the endogenous Id-1 promoter (McAllister, Christian, Horowitz, Garcia, & Desprez, 2007) and indirectly through the upregulation of extracellular signal-regulated kinase (ERK), which subsequently leads

to a downregulation of the Id-1 expression. Additionally, the pro-differential factor Id-2 is upregulated. Overall, these modulations result in a significantly smaller primary tumor and a reduction in size and number of metastatic foci (McAllister et al., 2011). Moreover, the cell death in breast cancer cells has been analyzed in detail. Both apoptosis and autophagy are inducted by CBD, promoted through various pathways aiming at the induction of endoplasmic reticulum (ER) stress and inhibiting AKT/mTOR signaling (Shrivastava et al., 2011).

Beside breast cancer, CBD has not failed to show its antitumor properties in numerous other cancer types. In glioma cell lines, *in vivo* and *in vitro* studies showed the antineoplastic activity of the cannabinoid. The antiproliferative effect could be reduced with the antioxidant agent α-tocopherol, suggesting that oxidative stress could be a part of the cell growth inhibiting mechanism (Massi et al., 2004). Furthermore, a downregulation of the Id-1 expression has been observed in glioma cells as well, a pathway that regulates the aggressiveness of the tumor. The lower level of Id-1 led to a significant inhibition of cell invasion, similar to the effect registered in breast cancer cells (McAllister et al., 2007; Soroceanu et al., 2013). Considering a systematic review on overall 35 studies about the antitumor effects of cannabinoids on glioma cells, all included studies registered antineoplastic effects, whereas apoptosis and cell cycle arrest were two common types of cell death. Still, one must take notice that not only CBD but various other cannabinoids were reviewed in the meta-analysis (Rocha, Dos Santos Junior, Stefano, & da Silveira, 2014).

Apart from breast and brain cancer cells, also lung cancer cells have shown to be sensitive against the effects of CBD (Misri et al., 2022). The intercellular adhesion molecule 1 (ICAM-1) in primary lung cancer cells is upregulated through CBD, causing an increased cancer cell lysis by lymphokine-activated killer (LAK) cells (Haustein, Ramer, Linnebacher, Manda, & Hinz, 2014). Furthermore, CBD-induced apoptosis in CRC cell lines has been analyzed. Noxa, which is a pro-apoptotic member of the Bcl-2 family (Ola, Nawaz, & Ahsan, 2011), was observed to play an important role in the monitored cell death. CBD is responsible for reactive oxygen species (ROS) overproduction, which is linked with increased Noxa levels, eventually leading to apoptosis (Jeong et al., 2019). In melanoma models, an inhibition in viability, proliferation and tumor growth as well as an increase in apoptosis and autophagy was demonstrated after the treatment with a Sativex®-like drug consisting of equal amounts of THC and CBD (Armstrong et al., 2015). Other tumors that have proved to

be sensitive to the antitumor activity of CBD are pancreatic (Sharafi, He, & Nikfarjam, 2019) and prostate cancer (De Petrocellis et al., 2013).

As is well known, *in vitro* studies cannot always be transferred 1:1 to clinical medicine, making clinical experience with cancer patients of great importance. Recently published case series of patients with brain cancer indicate a therapeutic effect in most patients (Likar, Köstenberger, & Nahler, 2020), but further systematic clinical studies with CBD are yet to be carried out.

When observing non-tumor cell lines such as HaCat (human keratinocyte), 3T3-F442A (rat preadipocytes), and RAW 264.7 (mouse monocytemacrophages), the vitality was not affected after CBD treatment of a dose similar to the concentration exerting 50% inhibition (IC_{50}) in the tested breast cancer cell lines (Ligresti et al., 2006). Also, normal colorectal tissue cells did not show an impact on their proliferation after CBD treatment in contrast to CRC cells (Jeong et al., 2019). This data indicates that CBD attacks malignantly transformed cells rather than normal body cells, making it a particularly safe drug in an oncological setting.

In summary, the non-psychoactive drug CBD can be considered as a promising and safe therapeutic agent in cancer therapy, but further research in form of clinical trials still have to be made.

1.3. Heat shock protein 70 (Hsp70)

1.3.1. Overview

The Heat shock proteins (HSPs) were first described in 1962, when Ritossa investigated that a temperature elevation in *Drosophila melanogaster* leads to a heat shock response involving a higher expression of the HSP coding genes. HSPs consist of a large family of proteins that are classified according to their molecular weight: Hsp40, Hsp60, Hsp70, Hsp90, etc. (Li & Srivastava, 2004). The 70-kDa HSP family (Hsp70) comprises 13 members, which can be categorized by their subcellular location and their reaction to stress stimuli. Generally, a distinction is made between the constitutively expressed heat shock cognate protein 70 (Hsc70) and the major stress inducible heat shock protein 70 (Hsp70) (Murphy, 2013; Radons, 2016). These proteins are highly conserved across various species from bacteria over plants to animals, meaning the coding nucleotide sequence remained nearly unchanged during

evolution (Lindquist & Craig, 1988). They also exist under physiological circumstances and are responsible for numerous cell protecting mechanisms (Jindal, 1996; Lindquist & Craig, 1988). Although heat is a major and also the name-giving inductor, several other stress factors have been investigated to upregulate the expression of the proteins as well. Anoxia, methanol, heavy metals, infections, inflammation and irradiation add to the list of known stimuli (Jindal, 1996; Kalmar & Greensmith, 2009; Lindquist & Craig, 1988). In the following, particularly the Hsp70 family is elaborated on with regard to its structure, function and role in tumor cells.

1.3.2. Structure and Function

Hsp70 proteins consist of two major domains: the N-terminal nucleotide binding domain which has ATPase activity and the C-terminal substrate recognition domain which binds unfolded proteins (Flaherty, DeLuca-Flaherty, & McKay, 1990). They are located in most cellular compartments, such as cytosol, endoplasmic reticulum (ER), mitochondria, nucleus, nucleolus and the cell membrane (Multhoff & Botzler, 1998). Hsp70 proteins are called ‘molecular chaperones’ as they play a crucial role in the folding of nascent polypeptides. Moreover, they are responsible for the import of proteins into cell organelles and prevention of protein aggregation (Hartl, 1996; Radons, 2016).

When located extracellular or on the cell surface, Hsp70 plays a major role in the immune response. The protein can form complexes with antigens which antigen presenting cells (APCs) can detect via Hsp receptors. After phagocytosis, the fragments can be presented to the CD8+ cytotoxic T cells through major histocompatibility complex (MHC) class I and therefore induce an immune response (Basu & Srivastava, 2000; Srivastava, 1994; Suto & Srivastava, 1995). Exogenous Hsp70 also stimulates the innate immune system as it promotes the expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 after a specific surface binding to human monocytes (Asea et al., 2000; Vostakolaei et al., 2021). Another mechanism of Hsp70 eliciting the immune response is based on the fact that natural killer (NK) cells recognize membrane bound Hsp70. Normal body cells only show intracellular Hsp70, but tumor cells (Multhoff et al., 1995) and also other targets of the immune system such as virally infected cells express membrane bound Hsp70 (mHsp70) (Moseley, 2000). NK cells are able to induce a perforin-independent, granzyme B-

mediated apoptosis in the malignantly transformed and infected body cells (Gross, Koelch, DeMaio, Arispe, & Multhoff, 2003; Vostakolaei et al., 2021).

Additionally, Hsp70 proteins show anti-apoptotic effects in the cell. They inhibit the activation of Bcl-2 family member Bax and reduce the permeability of the mitochondrial membrane, hindering the mitochondria to release its pro-apoptotic factors (Radons, 2016; Stankiewicz, Lachapelle, Foo, Radicioni, & Mosser, 2005). Another mechanism Hsp70 uses to block the apoptosis is through binding to the apoptosis protease-activating factor 1 (Apaf-1) and therefore inhibiting the formation of the apoptosome (Beere et al., 2000).

1.3.3. Role of Hsp70 in Tumor Cells

As mentioned above, tumor cells frequently show membrane bound Hsp70, in contrast to normal body cells, in which the protein is exclusively located intracellular (Multhoff et al., 1995). This phenomenon has been observed in a variety of different tumor entities such as colorectal, gastric, pancreas, head and neck, squamous cell lung carcinomas and hematological malignancies (Hantschel et al., 2000; Kleinjung et al., 2003; Pfister et al., 2007; Vostakolaei et al., 2021). As Hsp70 acts as a cell protector and anti-apoptotic agent it supports cell survival in cancer and therefore correlates with elevated tumor cell proliferation (Albakova, Armeev, Kanevskiy, Kovalenko, & Sapozhnikov, 2020). This suggests a poorer prognosis in patients with Hsp70 overexpressed cancers, which could be shown for colorectal (Hwang et al., 2003), breast (Ciocca et al., 1993), endometrial, uterine (Ciocca & Calderwood, 2005) and hepatocellular carcinoma (Chuma et al., 2003). However, this is not the case for every tumor entity that has been researched. For instance renal cancer even showed a better prognosis in patients with higher Hsp70 expression (Santarosa, Favaro, Quaia, & Galligioni, 1997). When only observing Hsp70-positivity versus Hsp70-negativity on the surface of tumor cells instead of the percentage of Hsp70-positive cells, Hsp70 can be regarded as a tumor-specific marker of aggressive disease. Lower rectal carcinoma and squamous cell carcinoma of the lung are associated with a less favorable prognosis if Hsp70-positivity was detected (Pfister et al., 2007).

As Hsp70 is expressed only on the membrane of tumor cells and the protein can be recognized through effector cells of the immune system, it identifies as a promising tumor-specific target structure. The cytolytic activity of NK cells against the malignantly transformed cells can be enhanced through the incubation with TKD and IL-2. TKD is

a 14-mer peptide from the substrate binding domain of Hsp70 and has shown to stimulate both the cytolytic and the proliferative activity of NK cells (Gastpar et al., 2004; Multhoff et al., 2001). After Krause and colleagues could demonstrate a high tolerability of *ex vivo* TKD/IL-2 stimulated, autologous NK cells following radiochemotherapy in patients with metastasized non-small cell lung cancer (NSCLC) in a phase I clinical study (Krause et al., 2004), a phase II clinical trial was conducted in 2014. Recent results show that the HSP-70 based therapy appears to be both safe and effective (Multhoff et al., 2020).

Tumor cells are able to regulate their mHsp70 expression after different stimuli. Both the membrane expression and the density of Hsp70 can be upregulated in carcinoma cell lines after irradiation (Gehrmann et al., 2005). Treatment with the chemotherapeutic agents vincristine and paclitaxel (Gehrmann et al., 2002), hypoxia (Schilling et al., 2009) and photodynamic therapy (Korbelik, Sun, & Cecic, 2005) also lead to an elevated mHsp70 expression in tumor cells. These factors are especially relevant, since many tumor treatment plans are based on these conditions. On the one hand, tumor cells have stronger protection against radiation- and chemotherapy-induced apoptosis through the upregulation. But on the other hand, membrane-bound Hsp70 represents a target structure for NK cells and the amount of Hsp70 shows a positive correlation with the NK cell-mediated cytolysis. Considering the enhanced tumor cell lysis, an upregulation might contribute to a favorable clinical outcome in immune competent or even immune stimulated patients (Albakova et al., 2020; Gehrmann, Radons, Molls, & Multhoff, 2008).

In summary, membrane bound Hsp70 is a tumor cell specific feature and is considered a promising target for cancer therapies.

1.4. Warburg-effect

In 1924, the physiologist Otto Warburg investigated that cancer cells metabolize glucose in a different manner to normal body cells (Otto Warburg, Posener, & Negelein, 1924). He found out that cancer cells prefer to generate their energy through fermentation of glucose into lactate instead of using the more efficient oxidative phosphorylation, even though oxygen is present (Vaupel & Multhoff, 2021). This inefficient way of energy production in cancer cells is called “aerobe glycolysis” (Vander Heiden, Cantley, & Thompson, 2009; O. Warburg, 1956). A definite

explanation for the different metabolism remains unclear, but Warburg's hypothesis that the Warburg-effect is the reason for carcinogenesis is considered obsolete (Vander Heiden et al., 2009).

2 Aim of this Study

Contemplating the incidence of cancer, colorectal cancer ranks third among the most common cancers. Although screening programs and effective treatment plans have been developed by now, colorectal cancer also takes up the third place in leading causes of cancer death (Siegel et al., 2021). Hence, more research in colorectal cancer therapy is needed.

The cannabinoid CBD has been known and studied for decades, as it is a major component of the prominent hemp plant (Adams et al., 1940). But the antitumor properties of this agent are only newly assessed. It has shown antineoplastic activity in several tumor cell lines, including colorectal (Jeong et al., 2019), lung (Haustein et al., 2014), breast (McAllister et al., 2011), pancreatic (Sharafi et al., 2019) and prostate cancer (De Petrocellis et al., 2013). However, more data about the mechanism and safety of the drug have to be gathered.

The aim of this thesis was to study the potential of CBD as a drug in colorectal cancer treatment. Therefore, the effect of CBD on colorectal tumor cells was analyzed and compared to the effect on normal body cells, as oncological treatment often is systemic.

Specific aims were to explore whether CBD acts as a Hsp70 inductor in tumor cells, as Hsp70 is known to show elevated expressions after stress stimuli (Lindquist & Craig, 1988). Another goal was to study whether different Hsp70 levels in tumor cells are associated with varying cytotoxic impacts of CBD.

3 Material

3.1. Chemicals and Reagents

Table 3.1: Chemicals and Reagents

Chemicals and Reagents	Company
Dimethyl sulfoxide (DMSO)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Methanol	Merck, Darmstadt, DE
Propidium iodide (PI)	Merck, Darmstadt, DE
Ethanol	BrüggemannAlcohol Heilbronn GmbH, DE
Cannabidiol 85705 250 mg Lot: 19585	PhytoLab GmbH, Verstenbergsgreuth

3.2. Solutions

Table 3.2: Solutions

Solutions and Buffers	Company
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Dulbecco's modified eagle's medium (DMEM)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
RPMI-1640 Medium (RPMI)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Fetal calf serum (FCS)/Fetal Bovine Serum	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
L-Glutamine (200 mM)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Sodium pyruvate (100 mM)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Penicillin/streptomycin (10,000 U/ml) PenNa, 10,000 µg/ml StrepSulfate)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Lymphocyte Separation Medium (LSM) 1077	Bio&SELL GmbH, Feucht / Nürnberg, DE
Trypan Blue (0.4%)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA
Trypsin/EDTA (0.05% Trypsin, 0.02% EDTA)	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA

Crystal violet solution 1%	Sigma life science, Sigma-Aldrich, St. Louis, MO, USA

3.3. Buffers

Table 3.3: Buffers

Buffer	Composition	Solvent	Application
Running buffer	15.1 g Tris 94 g Glycin, pH 8.3, 50 ml 10% SDS	dd	Gel electrophoresis
Transfer buffer	5.8 g Tris 2.9 g Glycin, pH 8.3, 200 ml Methanol, 3.7 ml 10% SDS	dd	Western Blot: transfer to membrane
TBS	2.42 g TRIS, pH 7.6, 0.8 g NaCl	dd	Western Blot
TBST	40 ml TBS, 500 µl Tween 20	dd	Western Blot: washing
Blocking buffer	5% Milk powder	TBST	Western Blot: blocking
RIPA buffer	150 mM NaCl, 1 % Triton X-100, 50mM Tris pH 8.0, 0.5% Sodium deoxycholate, 0.1% SDS		Cell lysates

3.4. Proteins and Antibodies

Table 3.4: Proteins and Antibodies

Antibody (Clone)	Host	Isotype	Conjugate	Company
Mouse IgG1 Lot 8160979	Mouse	IgG1	FITC	BD Biosciences, San Jose, CA, USA
cmHsp70.1 Lot 04/2013	Mouse	IgG1	FITC	Multimmune, Munich, DE
Mouse IgG1 Lot 1693428B	Mouse	IgG1	APC	Thermo Fisher, Waltham, MA, USA
CD45 Lot 1966219A	Mouse	IgG1	APC	Thermo Fisher, Waltham, MA, USA

3.5. Kits

Table 3.5: Kits

Kit	Company
Annexin V-FITC, Apoptosis Detection Kit	R&D Systems, Minneapolis, MN, USA
PE Active Caspase-3 Apoptosis Kit	BD Biosciences, San Jose, CA, USA
Pierce BCA Protein Assay Kit	Thermo Fisher, Waltham, MA, USA
Pierce ECL Detection Kit	Thermo Fisher, Waltham, MA, USA

3.6. Cell lines

3.6.1. Tumor Cell Line LS174T

The used tumor cell line LS174T is a human colorectal adenocarcinoma cell line from a female 58-year-old Caucasian and was purchased from ATCC. Both the wild type (WT) and a genetically modified version, which had a knockout in the enzymes lactate dehydrogenase A and B (LDH -/-), were used. The knockout in these enzymes was generated by the group of Professor Kreutz and kindly given as a gift (Ždralević et al., 2018).

3.6.2. Non-tumor Cells

Peripheral blood mononuclear cells (PBMCs) from three independent human probands were used as representatives for non-tumor cells.

4 Methods

4.1. Tumor Cells

4.1.1. Cell Culture

The human colon adenocarcinoma cell lines LS174T wild type (WT) and lactate dehydrogenase A and B knockout (LDH -/-) were cultivated in DMEM supplemented with 10 % FCS, 1 % penicillin/streptomycin, 1 % L-Glutamine and 1 % sodium-pyruvate. All cells were kept in standard conditions at 37 °C in 5 % CO_2 and 95 % humidity in 12.5 cm^2, 25 cm^2 or 75 cm^2 cell culture flasks in a Heracell^{TM} 240i CO_2 Incubator by Thermo Fisher. In regular intervals from three to five days, the cells were split as described in the following way: the medium was removed, and the flask was washed with PBS. To separate the adherent cells, Trypsin/EDTA was added and the cells were incubated at 37 °C for 2 min. After the cells were detached completely, cell culture medium was added to stop the protease activity of Trypsin/EDTA, and the suspension was resuspended evenly. The procedure of splitting the cells was performed under an ENVAIReco Safe Comfort Plus Bench. After determining the number of cells per ml medium, an appropriate number of cells were seeded in a new cell culture flask with fresh cell culture medium.

4.1.2. Cell Counting

The exact number of cells was determined with a Neubauer Counting Chamber. The following calculation was made:

Cells/ml = (cell number/number of counted quadrants) x 10000 x dilution factor

A small amount of 20-50 µl cell suspension was transferred in a 96 well plate and resuspended with an equal amount of trypan blue. Dead cells with a permeable cell membrane were marked blue with trypan blue, therefore only bright cells were counted as viable. All cells were counted on a Primovert microscope by Zeiss.

4.1.3. Cryoconservation

For permanent storage, the cells were cryopreserved in liquid nitrogen. After harvesting cells as described above, 1-2 x 10^6 cells have to be centrifuged (300 g) for 5 min and washed once by removing the supernatant. The cell pellet was solved in cold medium/FCS/DMSO (50/40/10 %). DMSO prevents crystallization in the cells. The cells were transferred into cryo tubes and continuously frozen in a -80 °C freezing container. After 24 h the cells were transferred into liquid nitrogen. Thawing has to be done quickly to minimize the cytotoxic effect of DMSO. After thawing, the cell suspension was transferred into a falcon filled with 10 ml cell culture medium and centrifuged (300 g) for 5 min at room temperature. The cell pellet was resuspended in 15 ml fresh cell culture medium and transferred into a 75 cm^2 cell culture flask. Since this process can cause stress in the cells, they were split two to four times before they were used in experiments.

4.1.4. Treatment with CBD

The cells were treated with different concentrations of CBD, which was dissolved in methanol. In order to exclude methanol as a confounder, CBD-treated samples were compared to samples treated with the vehicle methanol. The amount of methanol in the sham-sample was equal to the amount of methanol in the sample with the highest concentration of CBD. Concentrations of 1; 2; 5; 7.5; 10; 15 and 20 µM were used. The treated cells were always incubated for 48 h in standard conditions at 37 °C in 5 % CO_2 and 95 % humidity.

4.1.5. Colony Formation Assay

In order to determine the survival of the cell lines LS174T WT and LDH -/- after treatment with different CBD concentrations, a colony formation assay (CFA) or also called clonogenic assay was performed. The cells were harvested as described above in 4.1.1 and only a small number of cells, which was 800 for WT cells and 1800 for LDH -/- cells, was plated in a 12 well plate. To lower the error in the analysis, a row of three wells always got the same treatment meaning the same dose of CBD as illustrated in Figure 4.1. The plates were incubated under standard conditions at 37 °C in 5 % CO_2 and 95 % humidity for 7-8 days. After this time period, cell clones have

formed colonies of at least 50 cells in the untreated group. As a first step of the staining, the medium was removed, and the plates were washed once with PBS. The cells were fixed with 1 ml of -80 °C cold methanol for 5 min. After removing the methanol, the cells were stained with 1 ml 0.1 % crystal violet for 2 min. Finally, the plates were rinsed with distilled water and air-dried completely. The stained colonies were counted by an automatic counting bio reader. Therefore, customized measuring methods were established, making sure that only colonies consisting of 50 or more cells were counted. To determine the relative survival, the plating efficiency (PE) and the surviving fraction (SF) was calculated. The following equations were used:

PE = (number of colonies counted / number of cells seeded) x 100
SF = (number of colonies counted / (number of cells seeded x PE)) x 100

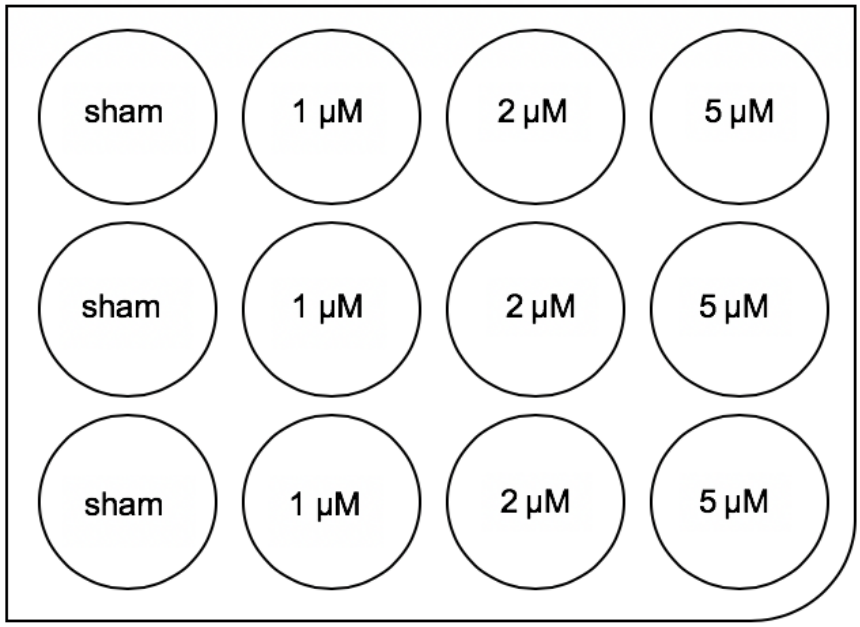

Figure 4.1: Treatment of cells in 12-well plate

4.1.6. mHsp70 Flow Cytometry

Flow cytometry or FACS analysis (fluorescence activated cell sorting) enables to analyze cells through their unique features, such as size, granularity and the expression of surface markers. The analysis is based on the optic signals which the cells emit while passing the laser of the machine. Every cell is lead separately through the laser with the help of a special pressure system. When the laser beam runs across the cell, scattered light is generated. The Forward Scatter (FSC) represents the size

of the cells and the Side Scatter (SSC) represents the granularity of the cells. Additionally, the surface markers of the cells are shown through a linkage of a dye and an antibody. The laser initiates the dye to emit a light of a certain wavelength which is specific for every dye (Adan, Alizada, Kiraz, Baran, & Nalbant, 2017; Delude, 2005; Shapiro & Telford, 2018). All samples were measured on a FACS Calibur.

In order to determine the unspecific binding of the antibody a FITC-bound isotype antibody was used as a negative control while MHC I was used as a positive control for human cells. Propidium iodide (PI) 0.02 mg/ml was used to exclude dead cells.
The membrane-bound Hsp70 on viable tumor cells was identified with the FITC-bound mouse monoclonal antibody cmHsp70.1 mAb, that was generated by the group of Prof. Multhoff (Stangl et al., 2011).

Cells were seeded with an appropriate cell number that lead to a confluence of 60-80 % after two days. Cells were harvested as described in 4.1.1. All steps were performed on ice. 0.2 x 10^6 cells were transferred into 1.5 ml micro tubes and centrifuged (300 g) for 5 min at 4 °C. Medium was removed and the cell pellet was resuspended with 1 ml cold FACS buffer containing PBS and 10% FCS and centrifuged (300 g) for 5 min at 4 °C. The supernatant was removed with a vacuum pump and the cmHsp70.1 antibody (1:20 in PBS) was added and resuspended. After an incubation time of 30 min on ice in the dark, the samples were washed with FACS buffer by centrifuging (300 g) for 5 min at 4 °C to remove unbound antibodies. The supernatant was removed with vacuum and 200-400 µl FACS buffer and 1 µl PI (0.02 mg/ml) per 100 µl FACS buffer were added and resuspended. The suspension was transferred into 5 ml FACS tubes and measured by the FACS Calibur instrument.

For the analysis, the software BD CellQuest Pro (BD Biosciences) was used. The data can be shown in dot plots, where every measured event is illustrated by a dot or through a histogram, where the intensity of the dye appears in a diagram. In an FSC vs SSC plot, a region of the requested subpopulation was created. This step allowed to analyze the cells of interest and to exclude cellular debris or other subpopulations. When portraying FSC vs. PI, a region of PI-negative, viable cells was analyzed. The percentage of positive stained cells was calculated as the number of specific-stained cells minus the number of unspecific-stained cells.

4.1.7. Pierce BCA Protein Assay

In order to quantify the total amount of protein for each required sample, the BCA Protein Assay was used. Therefore, cells were harvested as described in 4.1.1. and washed three times with PBS. Cell lysates were generated with RIPA buffer, which was incubated for 30 min. Samples were centrifuged at 12000 rpm for 20 min at 4 °C and the supernatant was transferred in new micro tubes. The Pierce BCA Protein Assay was used according to manufacturer's instructions to determine the total amount of protein for each sample.

4.1.8. Western Blot

As a first step, polyacrylamide gels were prepared. Table 4.1 shows the components for two 10% separating gels. After mixing together the required elements, the separating gel was covered with isopropanol and allowed to polymerize for 2 h. The isopropanol was removed, and the stacking gel was mixed according to Table 4.2 and added on top of the separating gel. Two combs were inserted, and the gel was left to polymerize for 1.5 h.

Table 4.1: Composition for two 10% Separating Gels

Component	Amount for two 10% separating gels
H_2O dd	7.9 ml
Acrylamide	6.7 ml
Tris pH 8.8	5.0 ml
SDS 10%	0.2 ml
APS 10%	0.2 ml
TEMED	8 µl

Table 4.2: Components for two 5% Stacking Gels

Component	Amount for two 5% stacking gels
H_2O dd	4.1 ml
Acrylamide	1.0 ml
Tris pH 6.8	0.75 ml
SDS 10%	60 µl
APS 10%	60 µl
TEMED	6 µl

After complete polymerization, the combs were removed, and the gels were transferred into the electrode chamber by Hoefer scientific instruments. Samples were mixed with loading buffer and heated at 95 °C for 10 min. The chamber was filled with running buffer and 20 µl of each sample was added. Gels were run at 80 V and 20 mA per gel for 2.5 h, leading to protein separation. In order to identify the proteins specifically, a semi-dry Western Blot system was used to transfer the proteins from the gel to a nitrocellulose membrane. Membrane, gel and four filter papers were soaked in transfer buffer and transferred in a Hoefer SemiPhor system by the following order: two filter papers, membrane, gel and two filter papers. Blotting was performed at 50 V and 60 mA per gel for 1.5 h. Subsequently, Ponceau staining was carried out. Therefore, Ponceau was applied on the membrane and afterwards washed with H_2O. Then, membranes were blocked with 5% milk powder in TBST for 1 h at room temperature. After removing the milk, the primary antibody (1:500 cmHsp70.1, diluted in 5% milk powder in TBST) was incubated over night at 4 °C. The next day the membranes were washed three times in TBST for 15 min and the secondary antibody (1:2000 Rabbit-anti-mouse) was incubated for 1 h at room temperature. After another three washing steps, Peroxide Solution (#1859701) was mixed 1:1 with Luminol Enhancer Solution (#1859698) and allowed to incubate on top of the membrane for 3 min at room temperature. Images were taken with the ChemiDoc™ Touch Imaging System by BIO-RAD.

4.1.9. Apoptosis Assays

In order to detect apoptotic cells, two different assays were performed: Annexin V FACS and caspase-3 FACS.

Annexin V binds to phosphatidylserine, which is flipped to the outer cell membrane in early apoptosis process (Sivagnanam, Palanirajan, & Gummadi, 2017). Since most of the dead cells are located in the medium of the cell culture flask instead of being adherent, the used medium was combined with the harvested cells and measured together. All steps were performed at room temperature, only just before measuring the cells were put on ice. 0.3×10^6 cells per tube were used and washed with cold PBS as described above. After removing the supernatant with vacuum, the cells were incubated with Annexin V-FITC and PI for 15 min at room temperature. Thereby it is possible to differ between early apoptotic cells with an intact cell membrane (Annexin V-FITC positive) and dead cells with a permeable cell membrane (PI positive). As

shown in Figure 4.2, the data are presented as dot plots. Four quadrants were analyzed: lower left represents viable cells (PI and Annexin V negative), lower right early apoptotic cells (Annexin V positive) and upper right late apoptotic or necrotic cells (PI and Annexin V positive). Untreated (A) and treated (B) samples were studied, meaning the percentage of cells per quadrant was compared. A positive control containing cells treated with 5 µM camptothecin for 4 hours was prepared.

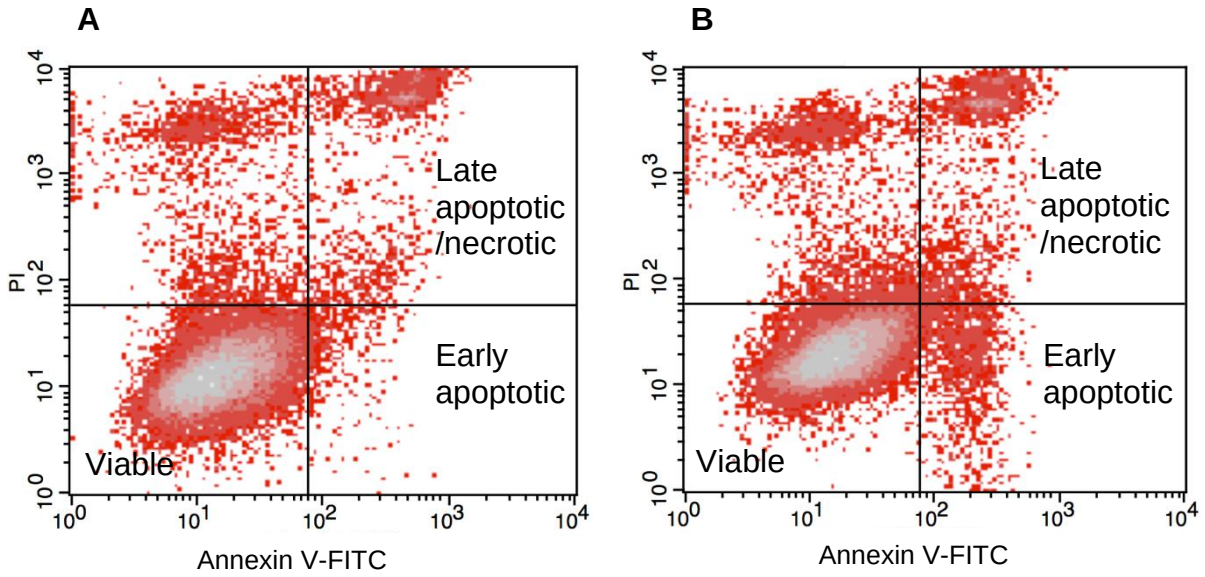

Figure 4.2: Flow cytometry analysis of LS174T cells using TACS Annecin V-FITC Apoptosis Detection Kit
Shown are the dot plots of untreated (A) and camptothecin-treated (B) cells.

Another method that was performed to detect apoptotic cells was the Caspase-3 FACS. The final stage of the process of apoptosis results in an activation of caspases, which are responsible for cellular breakdown. The executioner caspase-3 is only present in apoptosis and not in the other well-known form of cell death, necrosis (Denecker et al., 2001; Earnshaw, Martins, & Kaufmann, 1999).

As described above, many dead cells are located in the medium of the cell culture flask instead of being adherent, which resulted in combining the used medium with the harvested cells to enable a correct measurement. All steps were performed on ice. 0.3 x 10^6 cells per tube were used and washed with cold PBS as described above. After removing the supernatant with vacuum, the cells were incubated with Cytofix/Cytoperm solution for 20 min. The cells were washed with PBS twice and

incubated with an anti-caspase-3 antibody for 30 min at room temperature. After a final washing step, the cells were measured by the FACS Calibur instrument. In the analysis, the percentage of caspase-3 positive cells was defined as apoptotic and compared to caspase-3 negative cells. As mentioned above, a positive control with camptothecin-treated cells was always prepared.

4.2. Peripheral Blood Mononuclear Cells (PBMCs)

4.2.1. Density Gradient Separation of PBMCs

Blood samples were collected in EDTA tubes from three healthy donors of different age groups. When working with primary material such as blood, a separate laminar flow hood was used, determined for primary material exclusively. The blood volume was mixed with an equal amount of RPMI medium. In a new 50 ml falcon containing 10 ml of lymphocyte separation medium (LSM) with the density 1.077 g/ml at 20 °C, 30 ml of the blood RPMI mixture was carefully added on top of the LSM. Through the centrifugation on 2000 rpm at room temperature for 20 min, the two-phased mixture is separated into its different components. To prevent the mixture of the layers, a low acceleration (grade 4) and no deceleration was used. From the bottom to the top, the layers were separated into erythrocytes and dead cells, LSM and granulocytes, peripheral blood mononuclear cells (PBMCs) and the RPMI plasma mixture. The thin ring of PBMCs was extracted with a pipette and transferred in a new tube, RPMI medium was added and resuspended, then the tube was centrifuged (1800 rpm) for 15 min at 4 °C. After a second washing step the cell pellet was resuspended with 5 ml RPMI and counted like described above. To exclude erythrocytes in the cell suspension, acetic acid was used. Thereby only PBMCs are counted because any erythrocytes were destroyed through the acid. 2×10^6 PBMCs were pipetted in a well of a 6-well plate and kept in standard conditions at 37 °C in 5 % CO_2 and 95 % humidity. Stimulated lymphocytes were treated with IL-2 [100 IU/ml medium] for four days, as IL-2 is known to enhance the cell proliferation in T-cells (K. A. Smith, 1988).

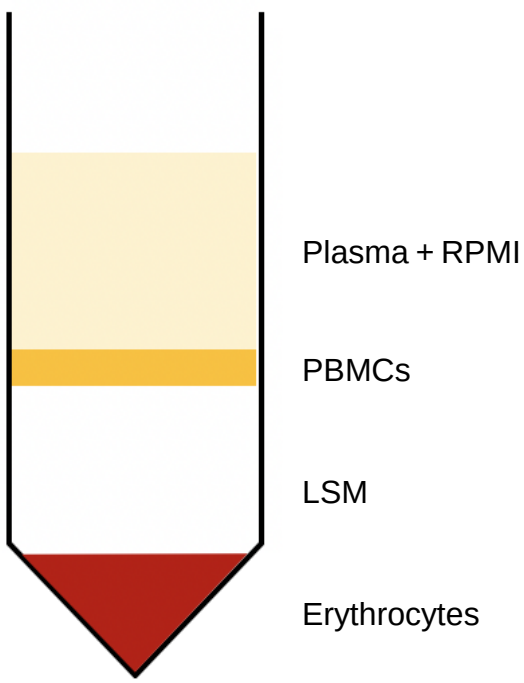

Figure 4.3: Cell layers after density centrifugation of blood with LSM

4.2.2. Flow Cytometry of Lymphocyte Markers

Peripheral blood mononuclear cells (PBMCs) were seeded six days prior to the experiment. The stimulated group was treated with IL-2 [180 IU/ml medium] for four days and CBD was added 48 h before the experiment. Cells were harvested as described in 4.1.1. Then, 0.2 x 10^6 cells were transferred into 1.5 ml micro tubes and centrifuged (300 g) for 5 min at room temperature. Medium was removed and the cell pellet was resuspended with 1 ml cold FACS buffer. After centrifugation, the supernatant was removed with vacuum and the APC-bound antibody CD45 was added and resuspended. After an incubation time of 15 min at room temperature in the dark, the samples were washed with FACS buffer by centrifuging (300 g) for 5 min at room temperature. This step enabled the removal of unbound antibodies. The supernatant was removed with vacuum and 200-400 µl FACS buffer and 1 µl PI (0.02 mg/ml) per 100 µl FACS buffer were added and resuspended. The suspension was transferred into 5 ml FACS tubes and measured by the FACS Calibur flow cytometer.

4.3. Statistical Analysis

The statistical analysis of the measured data was compiled with Microsoft Excel and SigmaPlot. All experiments were performed at least three times and the resulting means including the standard deviations are presented. For comparisons between two subgroups, the Student's t-test was applied. If more than two subgroups were compared, a two-way ANOVA with the Tukey method as post-hoc analysis was realized. The resulting p-value expresses the level of significance of the data.

Table 4.3: Level of Significance

Significance level	Presentation
$p < 0.05$	* (significant)
$p < 0.01$	** (very significant)
$p < 0.001$	*** (highly significant)

5 Results

5.1. Comparison of Tumor Cell Growth WT cells vs. LDH -/- cells

In the present study, the colon adenocarcinoma cell line LS174T wild type (WT) and the lactate dehydrogenase (LDH) A and B double knockout cell line (LDH -/-) were studied comparatively with respect to their growth behavior and sensitivity towards CBD. To prevent errors in the measurement of the Hsp70 expression or reaction to CBD which are caused by differences in the cell confluency, the growth behavior and the growth rate of both cell lines were determined. As illustrated in Figure 5.1 and Figure 5.2. the growth of the cell lines differed drastically. The WT cells showed an insular growth pattern which was in contrast to the rather diffuse growth pattern of the LDH -/- cells. Observing the cell proliferation of the two cell types, the mean doubling time for WT cells was about twofold higher compared to that of the LDH -/- cells (Ždralević et al., 2018). To obtain comparable growth rates, the LDH -/- cells were always seeded at a density which was twice as high as that of WT cells.

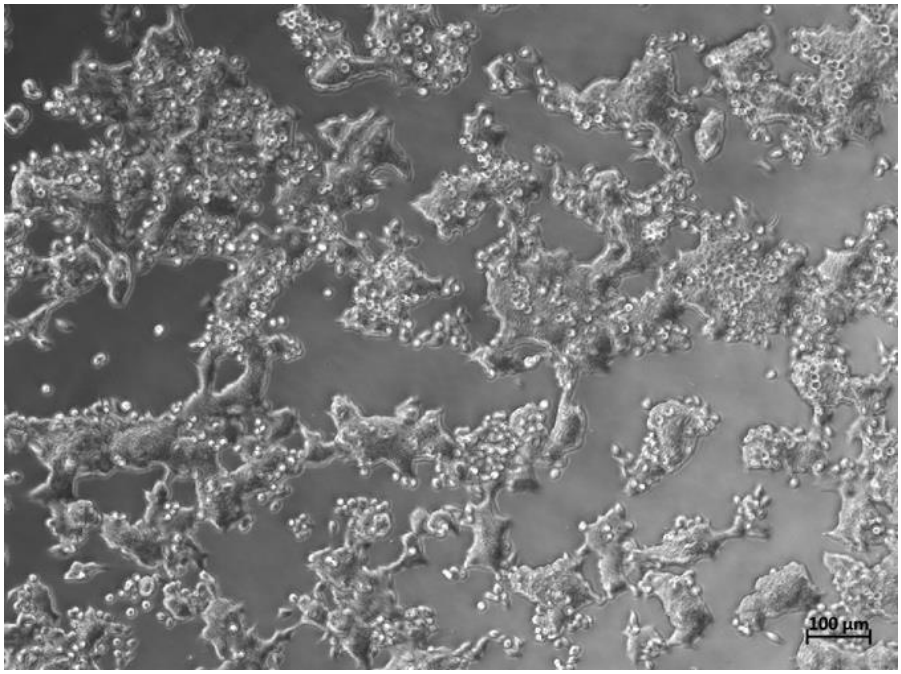

Figure 5.1: Growth pattern of LS174T WT cells, picture kindly provided by Melissa Schwab

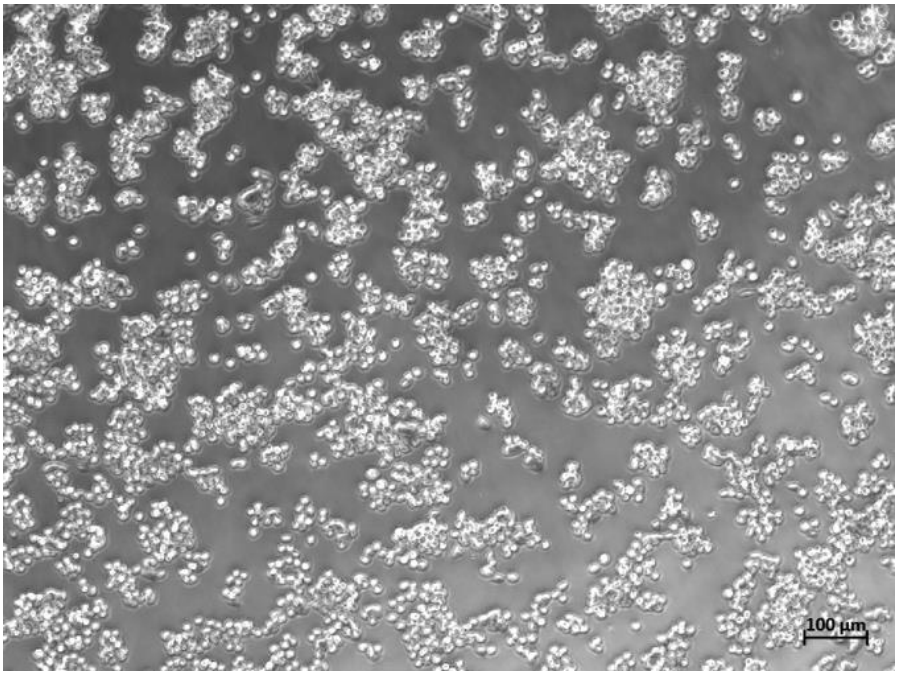

Figure 5.2: Growth pattern of LS174T LDH -/- cells, picture kindly provided by Melissa Schwab

5.2. Comparison of the mHsp70 Expression of WT cells vs. LDH -/- cells

Since many features of the knockout cell line appeared to be different to the WT cells (Ždralević et al., 2018), the mHsp70 expression of both subtypes were also compared. The membrane Hsp70 expression levels on WT cells were significantly ($p < 0.001$) higher than that of LDH -/- cells. As demonstrated in Figure 5.3, the mHsp70 expression on WT cells was 45.59 % ± 4.56 %, compared to LDH -/- cells that showed a mHsp70 expression only on 19.64 % ± 2.25 %.

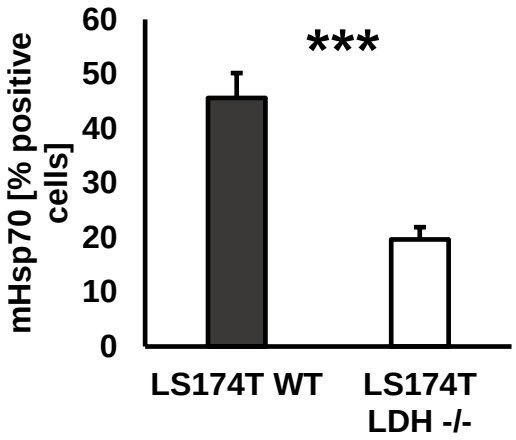

Figure 5.3: mHsp70 expression of WT cells vs. LDH -/- cells

5.3. Comparison of the Intracellular Hsp70 Expression of WT cells vs. LDH -/- cells

A Western Blot was performed to examine the intracellular Hsp70 expression in both tumor subtypes. Representative Western Blots are shown in Figure 5.4, all bands were normalized to the loading control β-Actin. When comparing the Hsp70 level of the LDH -/- cells to the WT cells, a significantly ($p < 0.001$) lower expression of the protein was measured. As shown in Figure 5.5, the level of Hsp70 in the LDH -/- cells was only 86.88 % ± 1.41 % of the level in the WT cells.

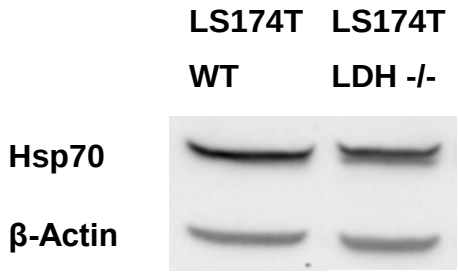

Figure 5.4: Representative Western Blot images of WT cells vs. LDH -/- cells

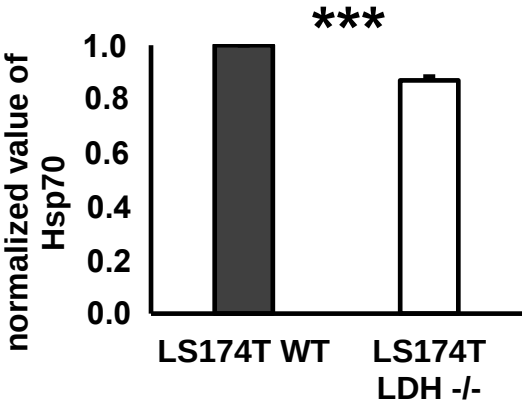

Figure 5.5: Intracellular Hsp70 expression in LS174 WT cells vs. LDH -/- cells

5.4. Effects of CBD on Tumor Cells

In order to assess the impact of CBD on tumor cells, tumor cells were exposed to CBD for 48 h or longer and several effects were examined. Initially, the Hsp70 expression on the membrane and in the cytosol were measured by flow cytometry and Western Blotting. Afterwards, the viability of the cells was studied by standard cell culture and colony formation assays and apoptosis was measured by flow cytometry.

5.4.1. mHsp70 Expression

Since Hsp70 is known to become upregulated by various stress stimuli (Jindal, 1996; Lindquist & Craig, 1988), it was examined whether CBD can cause similar effects. Flow cytometry was applied to analyze the mHsp70 expression after treatment with CBD. Concentrations of 1, 2, 5 and 10 µM were used and an incubation period of 48 h. The mHsp70 expression of both cell types was compared to a non-treated sample which is termed sham treated. As shown in Figure 5.6, there was no significant difference between the mHsp70 expressions at any dose.

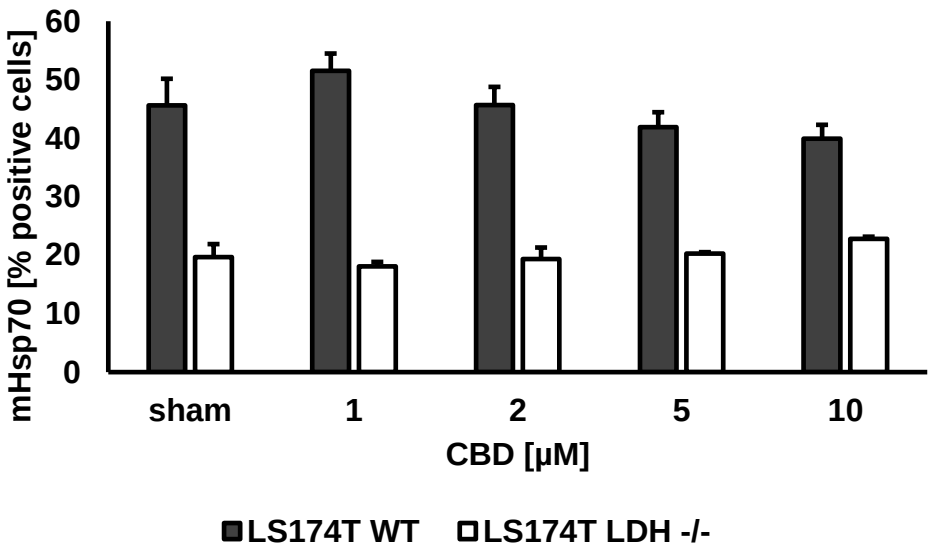

Figure 5.6: mHsp70 expression of LS174T WT cells and LDH-/- cells after treatment with CBD

5.4.2. Intracellular Hsp70 Expression

The effect of CBD on the expression of intracellular Hsp70 was examined. Therefore, a Western Blot was performed after exposure of the cells to CBD for 48 h. Bands were normalized to the loading control β-Actin, representative images shown in Figure 5.7. Concentrations of 5, 10 and 15 µM were used and compared to an untreated control sample. While the level of Hsp70 remained unaffected for the LDH -/- cells, the WT cells showed a dose-dependent upregulation of the protein (cf. Figure 5.8). The increase of Hsp70 was significant ($p < 0.01$) for the concentration of 10 µM, showing a 1.23-fold density change.

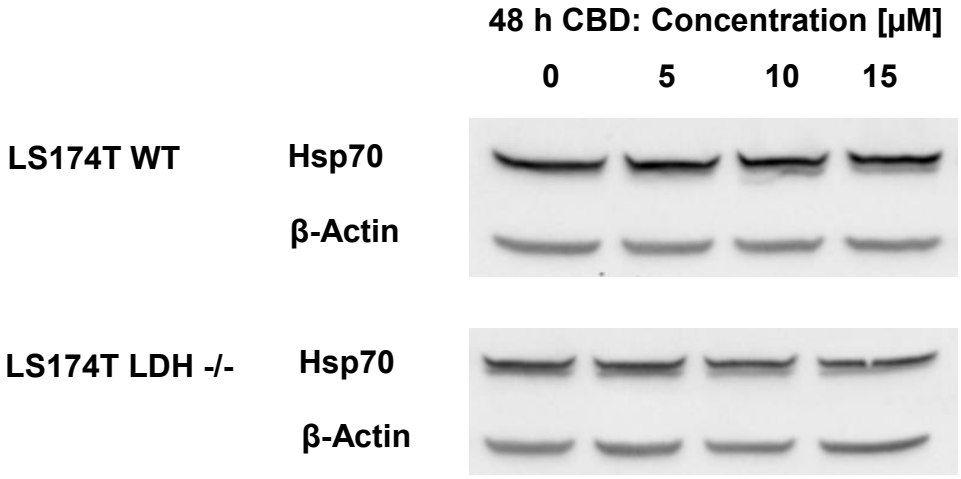

Figure 5.7: Representative Western Blot images of WT cells vs. LDH -/- cells

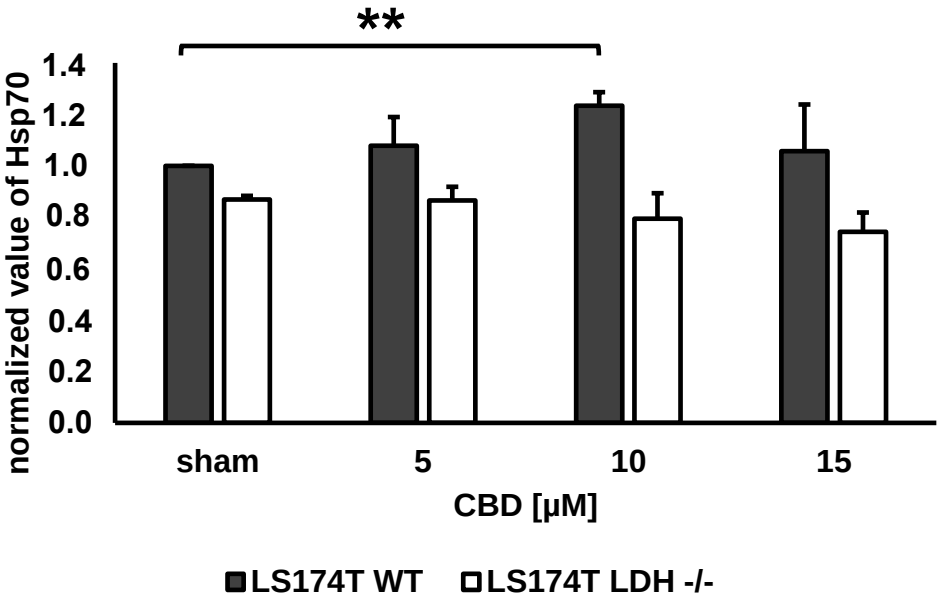

Figure 5.8: Intracellular Hsp70 expression of LS174T WT cells and LDH -/- cells after treatment with CBD

5.4.3. Viability in Standard Cell Culture

In order to evaluate the cytotoxic potential of CBD on tumor cells, standard cell culture assays were performed. Trypan blue was used to analyze the cell viability after exposure to CBD for 48 h. As presented in Figure 5.9, the viability decreased in a dose-dependent manner. At a concentration of 5 µM and above, the decrease in cell survival showed significant results for both WT and LDH -/- cells ($p < 0.05$). Concentrations of

10 µM and higher showed highly significant results ($p < 0.001$), as the relative viability declined to 54.34 % ± 10.64 % for the WT cells and 44.36 % ± 10.03 % for the LDH -/- cells. Interestingly, LDH -/- cells were significantly ($p < 0.05$) more sensitive to the drug compared to WT cells for concentrations of 15 and 20 µM of CBD.

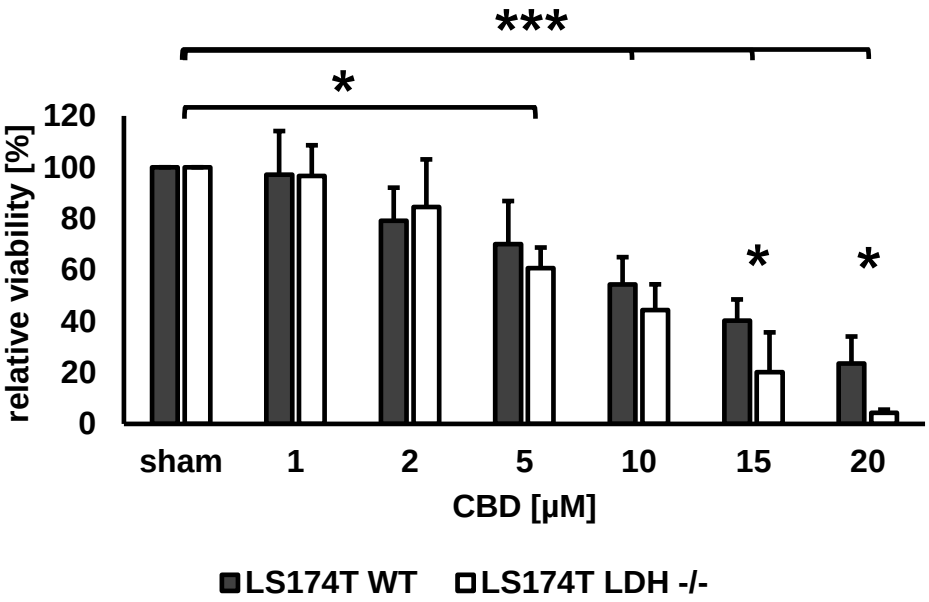

Figure 5.9: Relative viability of LS174T after CBD-treatment determined by trypan blue

5.4.4. Viability in Colony Formation Assay

Clonogenic cell survival assays (CFA) are the gold standard for measuring the survival of cancer cells *in vitro* after treatment with cytotoxic agents. A CFA was performed to analyze the effects of both cell lines after exposure to CBD. Figure 5.10 shows the survival fraction of LS174T WT and LDH -/- cells after CBD-treatment with doses ranging from 1 to 7.5 µM. A dose-dependent cell survival could be shown in both cell types, with highly significant ($p < 0.001$) results for concentrations of 5 µM and higher for WT cells and concentrations of 2 µM and higher for LDH -/- cells. Furthermore, it became clear that the knockout cell line was more sensitive to CBD compared to the WT cells. At a concentration of 2 µM CBD, the survival fraction of the LDH -/- cells was 0.51 ± 0.17, which was significantly ($p < 0.05$) lower to that of WT cells, with a value of 0.78 ± 0.13. The significance was even higher ($p < 0.001$) after treatment with 5 µM CBD, where the survival fraction of the LDH -/- cells declined to 0.13 ± 0.03 and the WT cells to 0.57 ± 0.12. From concentrations of 7.5 µM and higher, almost no survival

of the LDH -/- cells could be observed (0.03 ± 0.01), whereas the WT cells still showed few colonies (0.22 ± 0.12) ($p < 0.05$).

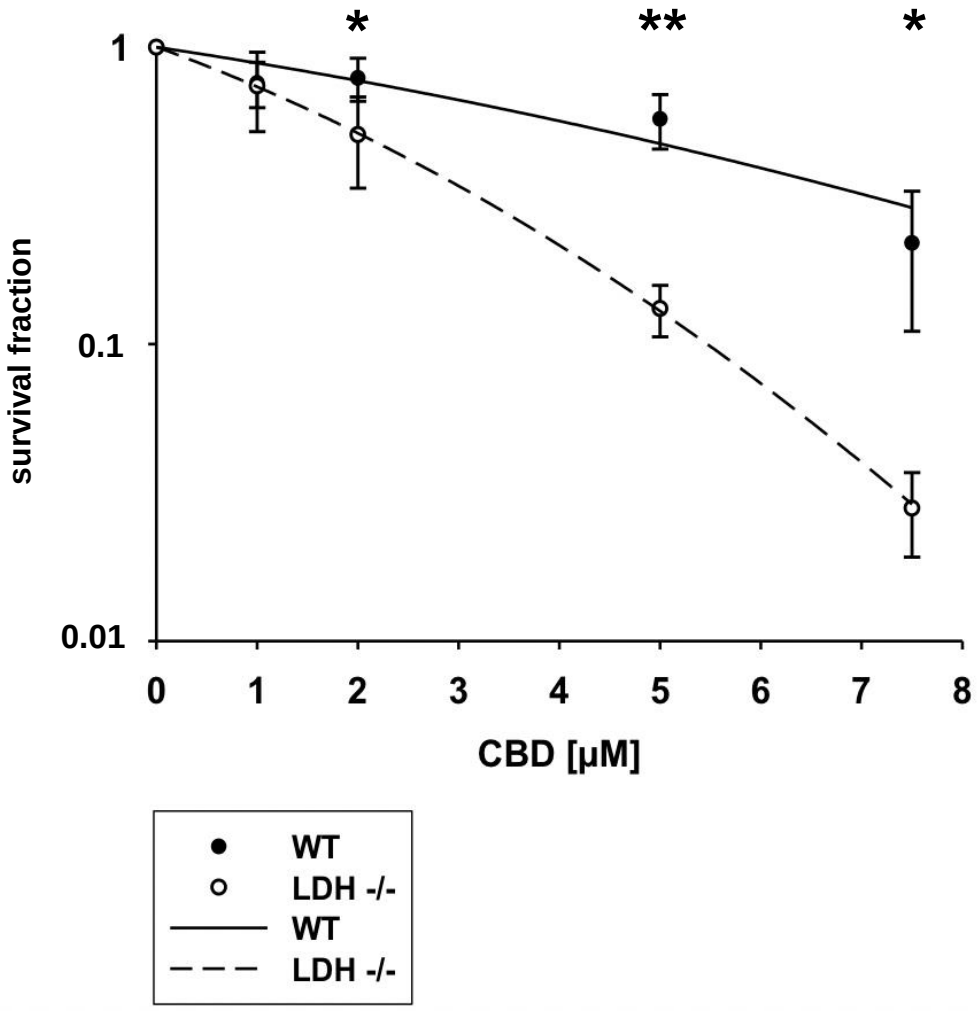

Figure 5.10: Survival fraction of LS174T WT cells vs. LDH -/- cells after CBD-treatment

5.4.5. Cell Death Analysis

In order to find out whether apoptosis was the mechanism of the cell death, an active caspase-3 FACS and an Annexin-V FACS were performed. Both methods showed no significant increase of apoptotic cells compared to the negative control.

5.5. Effects of CBD on Normal Cells

Finally, to examine the selectivity of the effects of CBD on tumor cells versus non-tumor cells, peripheral blood mononuclear cells (PBMCs) from three healthy human volunteers were also tested. To enable a comparison, the same concentrations of CBD

were used, and the cells were also incubated for 48 h. For every concentration of CBD, two samples were prepared: unstimulated and stimulated. The stimulated PBMCs were treated with 100 IU of IL-2 for four days, which excites a proliferation of the cells (K. A. Smith, 1988).

5.5.1. Viability of Unstimulated PBMCs

In order to investigate the viability of PBMCs after CBD-treatment, the cells were counted with trypan blue, which allowed the visualization of dead cells. Compared to the untreated sham control, none of the used concentrations was significantly increasing cell death in normal cells. As shown in Figure 5.11, even after exposure to concentrations of 20 µM, 89.16 % ± 7.72 % of the cells were still viable relative to the sham.

To support this statement, the cells were also analyzed by flow cytometry. Therefore, CD45 was used as a typical leukocyte marker. Figure 5.12 represents the percentage of PI-negative cells for various concentrations of CBD. Similar results as in the cell stain could be generated with this method, showing that only a non-significant trend of decrease in the viability was observed.

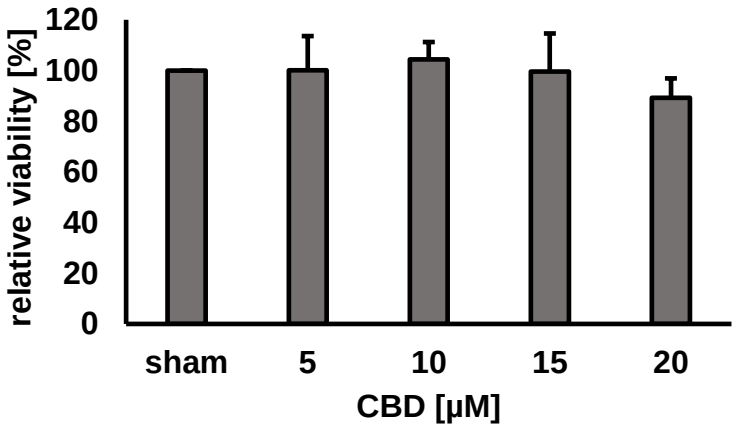

Figure 5.11: Relative viability of unstimulated PBMCs after CBD-treatment, measured with trypan blue

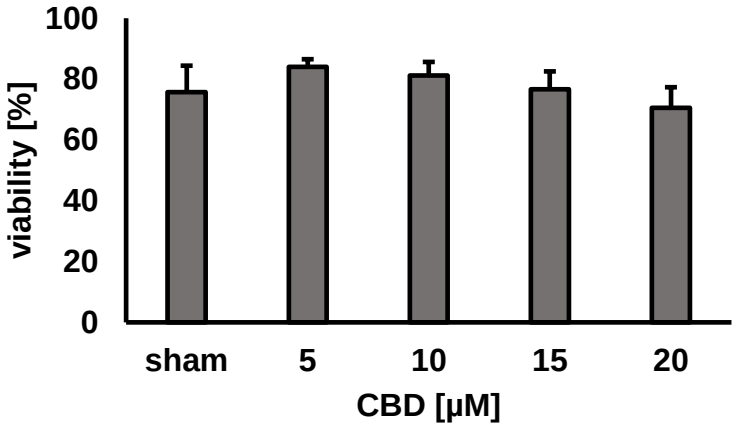

Figure 5.12: Relative viability of unstimulated PBMCs after CBD-treatment, measured with flow cytometry

5.5.2. Viability of Stimulated PBMCs

The same methods to assess the cell viability after exposure to CBD were applied for the stimulated PBMCs. Again, the cell dye exclusion test with trypan blue showed no significant increase in cell death with increasing concentrations of CBD (cf. Figure 5.13). The FACS-analysis confirmed the result showing similar data (cf. Figure 5.14).

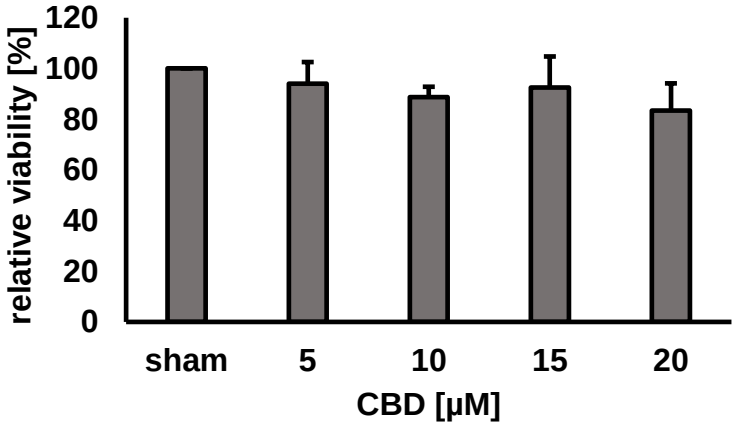

Figure 5.13: Relative viability of stimulated PBMCs after CBD-treatment, measured with trypan blue

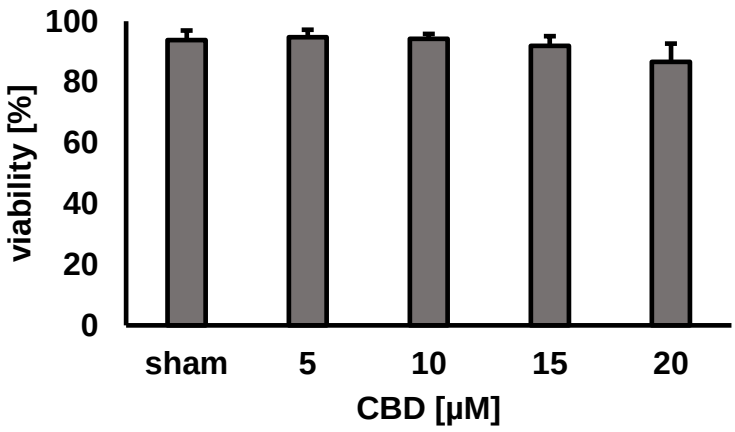

Figure 5.14: Relative viability of stimulated PBMCs after CBD-treatment, measured with flow cytometry

5.6. Summary of the Viability of Tumor versus Normal Cells

When comparing tumor cells with normal cells after CBD exposure, a clear difference in cell survival was observed. All tested concentrations (5 to 20 µM) of CBD showed highly significant ($p < 0.001$) results in the viability (cf. Figure 5.15).

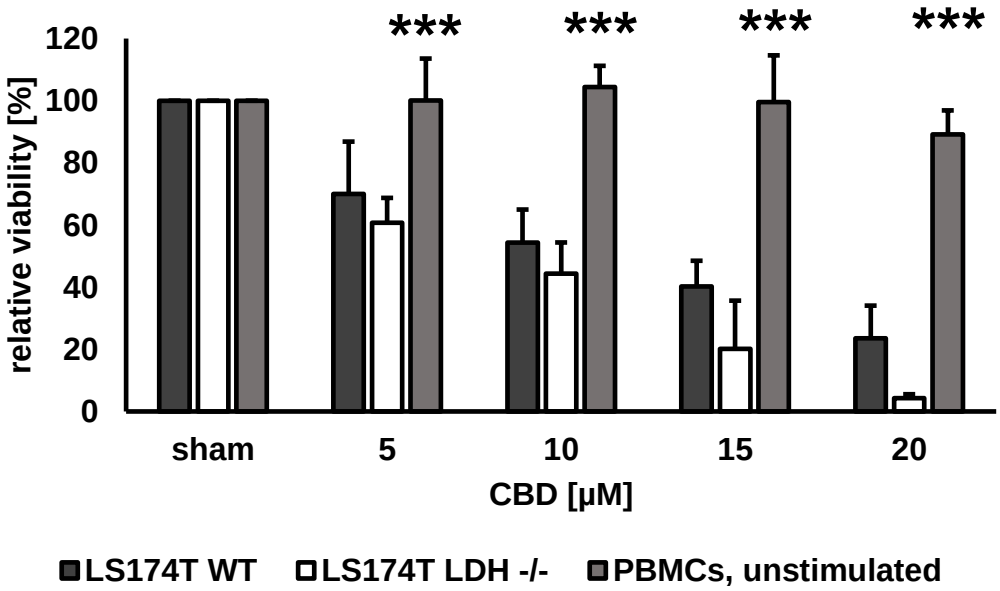

Figure 5.15: Comparison of the relative viability in tumor versus normal cells, determined by trypan blue

6 Discussion

The aim of this study was to investigate the potential of cannabidiol as a therapeutic agent in the treatment of colorectal cancer. To evaluate this question, the viability of colorectal cancer cells was examined after exposure to various concentrations of CBD. In order to assess the safety of the drug, the effects were compared to those on non-tumor cells. Additionally, the impact of CBD on Hsp70 was studied. Therefore, two different cell types of the colon carcinoma cell line with varying Hsp70 expressions were compared after treatment with CBD.

In the following, the role of Hsp70 is evaluated with regards to the ability of increasing the cytotoxic effects of CBD. Additionally, the metabolism in cancer cells is addressed and furthermore the CBD-mediated cell death is discussed. Finally, the potential of CBD in cancer therapy is examined in detail.

6.1. Hsp70 as a Potential Target to Enhance Cytotoxic Effects of CBD

Hsp70 is known to protect the cell and promote cell growth and survival (Jindal, 1996; Vostakolaei et al., 2021). The protein is upregulated after exposure to various cellular stressors including high temperature, inflammation and hypoxia (Lindquist & Craig, 1988). As a molecular chaperone, Hsp70 plays a crucial role in restoring homeostasis after cellular disturbance. Cancer cells can profit from this mechanism, as common therapy regimes like irradiation or chemotherapy increase the Hsp70 expression, which supports resistance (Boudesco, Cause, Jego, & Garrido, 2018; Brondani Da Rocha et al., 2004). Inhibition of Hsp70 thus presents an interesting therapeutic strategy in cancer research.

In the present work, the effects of CBD on the Hsp70 expression of tumor cells were investigated. Two cell types of the colon carcinoma cell line LS174T were examined: wild type and knockout with a double genetic disruption of the enzymes lactate dehydrogenase A and B. First of all, one must recognize that the LDH -/- cells express significantly ($p < 0.01$) lower levels of Hsp70 both on the membrane and in the cytosol. This implies that LDH -/- cells have reduced cell protection capacities compared to WT cells. In accordance with these findings, this study showed that the cytotoxic effects of

CBD have a significantly ($p < 0.01$) higher impact on the LDH -/- cells compared to the WT cells.

Scott and colleagues demonstrated that glioma cell lines expressed elevated Hsp70 levels following culture with CBD. Furthermore, they showed that the cytotoxicity of CBD was enhanced when combining the drug with HSP inhibitors (Scott, Dennis, Dalgleish, & Liu, 2015). Instead of glioma cells, this study examined the effect of CBD on colon carcinoma cells. Although the increase in Hsp70 was observed to a lesser extent, it could be confirmed that significantly elevated levels of Hsp70 were expressed in the cytosol of WT cells after CBD-treatment. This mechanism can support an improved survival. However, the CBD-mediated upregulation of Hsp70 was not measured in LDH -/- cells. The inability to increase the level of this protective protein could explain the higher sensitivity of the LDH -/- cells to the drug.

Moradi-Marjaneh and colleagues carried out a study in order to review the potential of Hsp70 inhibitors in colon carcinoma. Evaluating a large variety of agents that inhibit Hsp70 on different levels, some promising findings were demonstrated. Antitumor activities were observed in many cases, reducing the tumor size and improving responses to cancer treatment strategies (Moradi-Marjaneh, Paseban, & Moradi Marjaneh, 2019). The study of Schmitt and colleagues supports this thesis by demonstrating that the Hsp70 neutralizer ADD70 not only delays tumor growth but also sensitizes colon carcinoma cells in rats to the common chemotherapeutic agent cisplatin (Schmitt et al., 2006). When investigating the safety of HSP70 inhibitors, Barnoud and colleagues found out that the novel Hsp70 inhibitor AP-4-139B exhibits antineoplastic activity in colorectal cancer cells without appearing to be toxic to normal colon epithelial cells (Barnoud et al., 2020). Generally, the combinatorial treatment of Hsp70 inhibitors and anticancer drugs to synergistically enhance the cytotoxic effects is a well-studied model (French et al., 2013; Radons, 2016; Vostakolaei et al., 2021). These data suggest further experiments analyzing the role of Hsp70 inhibitors in combination with CBD in the colon carcinoma cell line LS174T.

In summary, lower Hsp70 levels seem to enhance the cytotoxic impact of CBD on tumor cells. It can be presumed that an upregulation of the protein reduces the effect of the cannabinoid. As increased levels of Hsp70 were measured in the tumor cells after exposure to CBD, further experiments after co-culture with Hsp70 inhibitors have to be done. Thereby the value of possible synergistic cytotoxic effects can be estimated.

6.2. Metabolism of Cancer Cells

The metabolism of cancer cells is characterized by an increased glucose consumption compared to normal cells. This circumstance occurs as a result of increased glycolysis and is maintained even under conditions with high oxygen; thus, their metabolism is described as aerobic glycolysis. This phenomenon is known as the Warburg effect (Kroemer & Pouyssegur, 2008; O. Warburg, 1956). Key enzyme of the Warburg effect is lactate dehydrogenase A (LDHA), which mainly reduces pyruvate to lactate. Lactate dehydrogenase B (LDHB) catalyzes the opposite reaction, lactate oxidation to pyruvate (Vander Heiden et al., 2009; Ždralević et al., 2018).

The used LS174T LDH -/- cell line was kindly gifted by the group of Professor Kreutz, that generated a double genetic disruption of LDHA and LDHB. The knockout resulted in a full suppression of lactate secretion and a reduction of glucose consumption. As a consequence, the metabolism of the cell line shifted to oxidative phosphorylation. The subsequent 2-fold reduction of the growth rate (Ždralević et al., 2018) could be confirmed by this study.

It is known that enhanced LDHA activity and accumulation of lactate can lead to resistance to chemotherapy (Hirschhaeuser, Sattler, & Mueller-Klieser, 2011). Furthermore, our group found out that inhibiting LDH activity in the colon carcinoma cell line LS174T not only leads to reduction in tumor cell growth but also to increased radiosensitivity due to decreased Hsp70-expression (Schwab et al., 2021). In line with this, the tumorigenicity of several types of cancers like lung (Xie et al., 2014), breast (S. Wang et al., 2021) and liver (Sheng et al., 2012) cancer can be decreased by targeting LDHA. This study also observed a significantly ($p < 0.01$) higher sensitivity of the genetically disrupted cells compared to the WT cells after treatment with the antitumor agent CBD. This suggests that the cytotoxic effects of CBD in cancer cells can be increased by therapeutic targeting of glycolysis.

However, it remains to be explored whether the knockout in the enzymes LDHA and LDHB and therefore inhibition of fermentative glycolysis or the low Hsp70 expression has a greater impact on the enhanced cytotoxicity of CBD on cancer cells.

6.3. Mechanism of Cell Death

This study could show that CBD induces cell death in cancer cells. However, the exact molecular mechanism of cell death is not well known. Several studies have reported

that apoptosis seems to play a major role in this context. Jeong and colleagues have found that CBD is responsible for reactive oxygen species (ROS) overproduction and subsequent apoptosis in CRC cells (Jeong et al., 2019). This is consistent with the findings of an *in vitro* human glioma study, where it was demonstrated that CBD triggers an early production of ROS followed by a caspase-activation in glioma cell lines (Massi et al., 2006). Other studies investigating the effects of CBD on lung cancer (Misri et al., 2022; Ramer et al., 2013), endometrial cancer (Fonseca, Correia-da-Silva, & Teixeira, 2018), prostate cancer (De Petrocellis et al., 2013) and leukemia (McKallip et al., 2006) have also reported that the main mechanism of cell death is regulated through apoptosis. It might be regarded as surprising that this study could not detect a CBD-mediated induction of apoptosis. This result was confirmed with two independent apoptosis assays, suggesting that the mechanism of cell death is different in the LS174T cell line.

However, ROS can not only induce apoptosis, but also autophagy (Chen & Gibson, 2008). A breast cancer study by Shrivastava and colleagues showed that both types of cell death are present after exposure to CBD: apoptosis and autophagy. Further research revealed the mechanisms of inducing the programmed cell death, which include ROS generation, ER stress and inhibition of mTOR signaling (Shrivastava et al., 2011). Although several components of pathways that are influenced by CBD have been revealed, the exact molecular mechanism is yet to be explored. Currently, data is not clear whether the CBD-mediated cancer cell death is connected to direct interactions with the cannabinoid receptors CB_1 and CB_2 or transient receptor potential cation channel subfamily V member 1(TRPV1), although most studies indicate a CB-independent mechanism (Fonseca et al., 2018; McAllister, Soroceanu, & Desprez, 2015). Additionally, several studies have shown that the ion channel transient receptor potential cation channel subfamily V member 2 (TRPV2) is triggered by CBD (Elbaz et al., 2018). Misri and colleagues could demonstrate the inhibition of tumorigenesis by CBD in cisplatin-resistant NSCLC cells through induction of apoptosis via TRPV2 (Misri et al., 2022). In glioma cells, also transient receptor potential cation channel subfamily V member 4 (TRPV4) has been found to be a target in CBD-mediated cell death (Huang et al., 2021).

The antineoplastic effects of CBD have appeared through various receptors, indicating a need for more research to elucidate the exact molecular pathways mediated by CBD.

6.4. Therapeutic Potential of CBD in Cancer Therapy

Cannabidiol is known to complement oncological treatment, as it has proven to benefit cancer-associated pain (Fraguas-Sánchez & Torres-Suárez, 2018). However, not only cancer-related symptoms, but also the tumor growth itself can be reduced by CBD. Several studies have examined the antineoplastic activity of the drug, showing promising results for breast (Fraguas-Sánchez et al., 2020; Ligresti et al., 2006), lung (Ramer, Fischer, Haustein, Manda, & Hinz, 2014) and brain (Huang et al., 2021; Massi et al., 2004) cancer.

This study also investigated the antitumor activity of CBD and compared the effects to non-tumor cells. The influence of CBD on normal tissue is an important factor to analyze in order to assess the safety of the drug. The colon carcinoma cell line LS174T was examined, as this cell line has not been studied before with regards to the effects of CBD. As representatives for non-tumoral cells, peripheral blood mononuclear cells from three healthy human volunteers were studied. PBMCs include blood cells with a round nucleus, meaning lymphocytes (NK cells, B cells and T cells), monocytes, and dendritic cells (Kleiveland, 2015).

CBD has shown its antineoplastic activity in various types of tumors. Particularly glioma cell lines are well researched, as many studies demonstrate CBD-mediated cytotoxicity *in vivo* and *in vitro* (Huang et al., 2021; Rocha et al., 2014). This is consistent with the findings of Ligresti and colleagues, who showed growth-inhibitory actions of CBD for a highly malignant human breast carcinoma cell line (Ligresti et al., 2006). Other tumors that have proved to be compromised by the cannabinoid are lung (Haustein et al., 2014; Misri et al., 2022) and prostate cancer (De Petrocellis et al., 2013). In accordance with these findings, this study showed a dose-dependent growth inhibition of CRC cells induced by CBD. Notably, Raup-Konsavage and colleagues were unable to detect significantly reduced cell viability in the CRC cell line LS174 after treatment with CBD (Raup-Konsavage et al., 2018). However, viability of LS174T cells was determined with the MTS assay in this study. The MTS test may be unsuitable to assess the cytotoxic impact of CBD, since other studies using different killing assays are showing different results. The reason for these contradictory results might be due to the fact that the exact mechanism how CBD kills tumor cells has not yet been elucidated completely.

Interestingly, non-tumor cell lines such as normal colorectal tissue (Jeong et al., 2019) or human keratinocytes (Ligresti et al., 2006) seem to be relatively insensitive to

the drug. Thus, these data indicate a tumor selective toxicity of CBD. Supporting this thesis, this study demonstrated that the survival of PBMCs was not affected after exposure to CBD. Already low concentrations such as 5 µM showed highly significant ($p < 0.001$) results when comparing the viability of tumor cells to PBMCs. Surprisingly, even the highest studied concentration of 20 µM did not affect the PBMCs significantly compared to the untreated control. At this dose, the tumor growth decreased to less than a quarter for WT cells and almost no growth at all for LDH -/- cells. In order to assess the selectivity of CBD for proliferating versus non-proliferating cells, IL-2 stimulated and therefore growing PBMCs were analyzed. Interestingly, the viability of stimulated PBMCs was not significantly compromised by CBD. These findings support the thesis that CBD shows selective cytotoxicity against tumor cells. In line with this, Devi and colleagues observed no increase in cell death in human T lymphocytes upon treatment with CBD (Devi et al., 2022). Although contrary results were seen in another study, where a CBD-mediated enhancement of apoptosis was reported in normal lymphocytes (Wu et al., 2008). However, one must take notice that Wu et al. isolated lymphocytes from mice and this study investigated human cells only. Nevertheless, other primary immune cells like monocytes appear to be relatively insensitive to CBD, as demonstrated by Gallily and colleagues. They could show that the growth inhibitory effects of CBD are selective for malignantly transformed cells like human myeloid leukemia cells (Gallily et al., 2003). The fact that this study analyzed PBMCs, which include both lymphocytes and monocytes, could explain the contrary results to Wu and colleagues. However, this data indicates that CBD targets transformed cells rather than normal body cells, although additional healthy control cells should be further examined.

In summary, CBD induces cell death in cancer cells including CRC cells. This effect could not be observed for normal PBMCs. Hence, the CBD-mediated cytotoxicity might be selective for tumor cells. According to that, CBD can be considered as a promising alternative therapeutic agent in the treatment of CRC.

Summary

Colorectal cancer ranks third among the most common cancers and also belongs to the three most common causes of cancer death. Cannabidiol (CBD), one of the main components of the hemp plant *Cannabis sativa*, shows antineoplastic properties. However, more data about the exact molecular mechanism and the safety of the drug need to be gathered. The aim of this study was to investigate the potential of CBD as a drug in colorectal cancer treatment. Furthermore, it was studied whether CBD exhibits increased antitumor activities on cancer cell lines with low expressions of the cell protecting heat shock protein 70 (Hsp70). Hsp70 plays an essential role in restoring homeostasis after cellular disturbance and can be upregulated through various stress stimuli.

For this purpose, two cell types of the colon carcinoma cell line LS174T were examined: wild type (WT) and knockout with a double genetic disruption of the enzymes lactate dehydrogenase A and B (LDH -/-). LDH -/- cells showed significantly lower Hsp70 values on the membrane and in the cell compared to WT cells. Following culture with CBD, a significant upregulation of intracellular Hsp70 was found in WT cells, but not in LDH-/- cells. Furthermore, a significantly higher sensitivity to the antitumor activity of CBD was revealed for the LDH -/- cells. These data indicate that the cytotoxic effects of CBD can be impeded by Hsp70.

In order to assess the safety of CBD, both tumoral and non-tumoral cells were exposed to concentrations of 1-20 µM. A dose-dependent growth inhibition could be observed for the tumor cell line. Already the low concentration of 5 µM induced a significant reduction in the cell survival compared to the untreated control. Peripheral blood mononuclear cells (PBMCs) from three healthy human volunteers were tested as non-tumoral cells. Interestingly, not even the highest studied concentration (20 µM) compromised the viability of the PBMCs. Surprisingly, even Interleukin-2 stimulated, proliferating PBMCs were not affected by CBD. Thus, the CBD-mediated cytotoxicity can be considered as selective for tumor cells. According to that, CBD might be a promising and safe alternative therapeutic agent in the treatment of CRC.

List of Figures

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