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IDENTIFICATION AND STUDY OF MODULATORS OF ANTICANCER THERAPY BASED ON PROTEOTOXIC STRESS

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Statement:

I declare that I wrote this thesis entitled „Identification and study of modulators of anticancer therapy based on proteotoxic stress" by myself. All references used for the writing of the thesis are included. The experimental part was carried out at the Institute of Molecular and Translational Medicine, Laboratory of Genome Integrity in Olomouc.

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Abstract

Proteotoxic stress (PS) is induced by damaged, unfolded, or aggregated proteins, which accumulate when the cell's chaperone or degradative capacity is overwhelmed. Defects in protein homeostasis are a typical phenomenon accompanying cancer cells, offering an effective therapeutic strategy as cancer cells are inevitably more vulnerable to PS due to accelerated protein requirements and increased aberrant protein production. Indeed, there are clinically used anticancer drugs targeting proteostasis. Interestingly, the anticancer effects of Disulfiram (DSF) - a drug initially developed to treat alcoholism which is now a hot candidate for oncology repurposing, are also attributed to interference with proteostasis. DSF's anticancer properties are attributed to its metabolite, bis-diethyldithiocarbamate-copper complex (CuET). Mechanistically, CuET induces aggregation of NPL4, an essential cofactor of the so-called VCP/p97-UFD1-NPL4 transferase complex. The complex is indispensable for processing proteins fixed in protein complexes and cellular structures. Thus, disulfiram, via its metabolite CuET represents a potentially effective and readily available anticancer drug. However, despite mechanistic rationale, multiple case reports, and promising preclinical results, disulfiram has shown less impressive results in clinical trials. One possible causation is explained in this work involving unaware drug-drug interaction. Namely, cancer patients often take various supplements to improve their therapy outcome or to attenuate the side effects. Unfortunately, they are introducing potential unwanted drug-drug interactions, which might decrease treatment efficacy. Such an effect is known for antioxidant dietary supplements hindering chemo-radiotherapy. Besides antioxidants, cannabis products have become very popular among cancer patients in recent years. Strikingly, in the search for modulators of CuET effectivity against cancer cells using a high-throughput screening approach, cannabidiol (CBD), one of the major cannabinoids present in a cannabis plant, scored as a potent CuET protectant. Detailed mechanistic insights presented in this work show that CBD induces the expression of metallothioneins, endogenous metal chelators, effectively protecting cells from CuET-mediated cytotoxic effects. This work might partially explain some of the inconclusive results of DSF’s clinical trials and simultaneously exposes CBD and cannabis-based products as potentially dangerous modulators of ongoing anticancer therapy.

Keywords: cannabidiol, metallothioneins, CuET, NPL4, protein aggregates, proteotoxic stress, drug resistance

Abstrakt

Proteotoxický stres (PS) je v buňkách způsoben poškozenými, chybně složenými nebo agregovanými proteiny. Ty se akumulují v okamžiku, kdy dojde k přetížení buněčných chaperonů a protein-degradačních mechanismů. PS patří mezi běžné jevy doprovázející rakovinu z důvodu zvýšené proteinové syntézy a akumulace aberantních proteinů. Cílení PS se tak nabízí jako efektivní strategie pro protinádorovou terapii. V současnosti existuje několik schválených léčiv interferujících s údržbou proteinové homeostázy a řada dalších je v klinickém testování. Jedním z nich je disulfiram (DSF) znám pod komerčním názvem Antabus. DSF je léčivo původně podávané k léčbě alkoholismu a nyní se stalo kandidátní látkou k léčbě rakoviny. Ukázalo se, že za jeho protinádorovými vlastnostmi stojí jeho metabolit CuET (molekula složená z jednoho iontu mědi a dvou molekul dietylditiokarbamátu). Mechanismus jeho účinku spočívá v agregaci a imobilizaci proteinu NPL4, nezbytného kofaktoru tvořící translokázový komplex s VCP/p97-UFD1. Celý komplex je nedílnou součástí údržby proteinů, které jsou například inkorporované do proteinových komplexů nebo pevných buněčných struktur. DSF tímto představuje potenciálně velmi efektivní protinádorovou terapii přímo cílící na proteinovou homeostázu. I přes to, že mechanismus působení je velmi účinný, což je podpořeno preklinickými studiemi i několika kazuistikami, výsledky v klinických protinádorových studiích nejsou úplně přesvědčivé. Jeden z možných důvodů je adresován v této práci. Onkologičtí pacienti se totiž snaží často podpořit protinádorovou léčbu, případně snížit její negativní účinky, užíváním různých terapeutických doplňků. Ty však mohou vést k nežádoucím lékovým interakcím, jak bylo dokázáno například pro antioxidanty ve spojení s chemo-radioterapií. Kromě různých antioxidantů jsou mezi onkologickými pacienty velmi populární i produkty z marihuany (*Cannabis sativa* L.). V této práci věnující se modulátorům biologické aktivity CuET, se za použití vysokokapacitního screenu podařilo identifikovat kanabidiol (CBD), jeden z převažujících členů kanabinoidních látek v marihuaně, jako látku interferující s biologickým účinkem CuET. Dále se podařil detailně objasnit mechanismus stojící za touto interferencí. Ukázalo se, že CBD vyvolává expresi metalothioneinů, endogenních chelátorů, které efektivně potlačují cytotoxický efekt CuET. Tato aktivita je pravděpodobně způsobena chelatací mědi z molekuly CuET. Výsledky práce tak mohou, alespoň částečně, vysvětlit nedostatečnost DSF v klinických studiích kde nebyla mapována exprese metalothioneinů ani souběžné užívání CBD. Zároveň varuje před CBD či marihuanovými produkty jakožto potenciálně nebezpečnými modulátory probíhající onkologické léčby.

Klíčová slova: kanabidiol, metalothioneiny, CuET, NPL4, proteinové agregáty, proteotoxický stress, léková resistence

Table of contents

1. INTRODUCTION

Proteotoxic stress belongs to hallmarks of cancer. The cancer cells produce an overdose of proteins, including an excess of aberrant ones. As a result, the cells are becoming extensively dependent on protein processing mechanisms. Under standard conditions, the proteotoxic stress regulates a cascade of responses associated with protein quality control mechanisms leading to refolding, sequestration, or degradation of particular proteins. In the case of an unmanageable situation, protein stress leads to programmed cell death, predominantly apoptosis [1].

Chemotherapy targeting protein homeostasis provides several FDA (American Food and Drug Administration office) options[2–4] (see chapter 1.4). Unfortunately, cancer treatment accompanies drug resistance. Nevertheless, many new drugs are developed and tested in clinical trials. One such medication is DSF which was initially designed to treat alcohol dependency as an aversion therapy, and currently, it has become a candidate for repurposing in cancer therapy[5–7]. Mechanistically, DSF is metabolised in the human body into many molecules, including copper-diethyldithiocarbamate complex (CuET). The complex targets Nuclear protein localisation protein 4 (NPL4), an integral part of the "Valosin-containing protein (VCP/p97)-Ubiquitin recognition factor in ER-associated degradation protein 1 (UFD1)-NPL4" complex acting as segregase in the ubiquitin-proteasome system (UPS)[8].

The drug resistance mentioned above may arise not only from canonical reasons such as the selection of cancer cells but also on the bases of patient supplementation with subsequent drug-drug interaction. It is already known that certain supplements, such as dietary antioxidants, may interfere with chemo-radiotherapy[9]. Patients with cancer are increasingly using cannabis products[10,11]. Cannabis is generally accepted as a plant with extraordinary beneficial effects, including anticancer properties. Due to legal aspects, a public market provides countless products based on cannabidiol (CBD), the non-psychoactive component of the cannabis plant[12]. Cannabis itself has been deeply studied for the last few years. The studies support a broad spectrum of targets modulating cell and tissue processes[12,13]. However, studies frequently exhibit significant variation in the methodology and subsequent findings, prompting cautious interpretation.

1.1. Ubiquitin proteasome system

Protein homeostasis is defined by dynamic processes composed of synthesis, folding, and degradation accompanied by strict control. Newly synthesised proteins undergo protein quality control based on identifying misfolded, toxic, dysfunctional, or immature proteins. The cells decide a protein's fate by choosing between degradation, sequestration, or refolding[14,15]. Before the degradation, proteins are tagged by regulatory polypeptides named poly-ubiquitin (Ub) chain acting as a signal for degradation in the 26S proteasome[16–18]. In addition to preserving protein homeostasis, the degradation process regulates numerous cellular processes, including the cell cycle, gene expression, apoptosis, carcinogenesis, etc.[19].

The UPS is an irreplaceable mechanism for the degradation of most proteins. The degradation process initiates with the binding of a poly-Ub chain. The modification is mediated by a family of E1, E2, and E3 enzymes[20–22]. Marked proteins are directed to the proteasome, where hydrolysation takes place and resulting short polypeptides are used as new building blocks for newly synthesised proteins[19,23,24]. The system is also linked with many assisting proteins responsible for the delivery of ubiquitinated proteins, their interaction with proteasomes or making the proteins accessible for degradation, see chapters 1.2 and 1.3.

Insufficiency of UPS's components leads to proteotoxic stress and is associated with various human diseases[25]. UPS is also essential for the survival of cancer cells, and thus it represents one of the targets for cancer therapy[26–29].

1.1.1. The ubiquitin code

In 1978, Ciehanover et al. (1978)[30] discovered a component linked to cellular proteolytic activity. The component was later identified as ubiquitin (Ub), a small protein with a molecular weight of up to 8,5 kDa. Ub is now known to be a residual molecule important for most cellular aspects, including[16–18,31]:

- proteasomal degradation
- protein homeostasis
- endocytosis

- receptor trafficking
- scaffolding
- selective autophagy
- cell signalling cascades
- DNA repair
- genome integrity
- epigenetic regulation
- cell cycle control
- programmed cell death

The meaning of the Ub signal is based on the location and architecture of the Ub mark, which is summarised under a single name: The ubiquitin code[16–18]. The signal could be monoubiquitination, multimonoubiquitination, or polyubiquitination. Polyubiquitination is divided according to the lysine (or methionine) on which the bond is formed (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63, and Met1). Except for homotypic chains, mixed and branched chains are presented (Fig. 1). Additionally, Ubs undergo further modifications. The example includes acetylation[17]. The plethora of possible combinations gives the Ub broad potential for application across cellular processes[17,32].

Ubiquitinating enzymes carry out the multi-step process of ubiquitination. The classification of enzymes is based on their function in a cascade. Ubiquitin is bound and transferred to an E2 Ub-conjugating enzyme by an E1 Ub-activating enzyme[20,21]. An E3 Ub-ligase ensures the transfer of Ub to a target substrate[22]. The opposite reaction, called de-ubiquitination, is mediated by deubiquitinases (DUBs)[18,33], see Fig. 1.

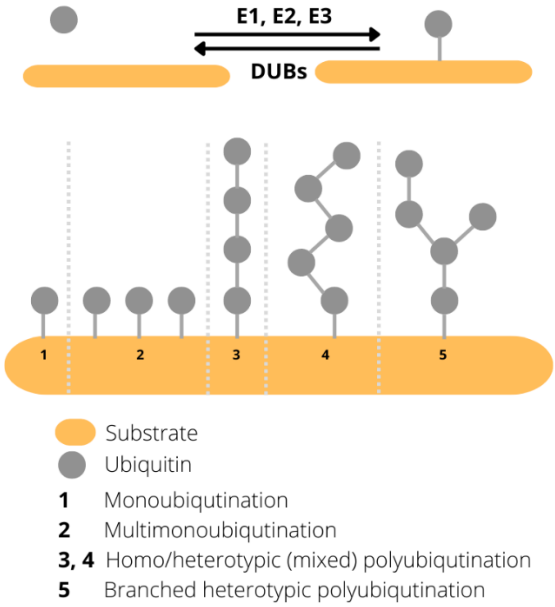

Fig. 1.: Schematic representation of the ubiquitin code together with enzymes mediating ubiquitination and deubiquitination: E1, E2 and E3, and DUBs.

Abbreviations: E1 – ubiquitin-activating enzyme; E2 – ubiquitin-conjugating enzyme, E3 – ubiquitin-protein ligase; DUBs – deubiquitinases

1.1.2. Proteasome

Protein degradation is mainly based on the interplay between UPS and the autophagy-lysosome system. Interestingly, autophagy is expendable under normal (nutrient-rich) conditions. Therefore, autophagy's primary function is recycling intracellular components to maintain energy metabolism and produce building material for new molecules[19,34]. Contrarily, proteasomal degradation is essential. Proteasome mediates the basal turnover of proteins, degradation of regulatory proteins, and those damaged or misfolded. Thus, the proteasome regulates the cell cycle, apoptosis, survival, metabolism, gene expression, and protein quality control and plays a crucial role in carcinogenesis and various diseases[19].

A 26S Proteasome is composed of a barrel-shaped 20S core particle and attached 19S regulatory particle from one or both sides (Fig. 2). In addition, the 19S regulatory particles could be replaced by alternative forms[19,23,24].

The 20S core particle, where the degradation takes place, contains α and β subunits forming a heteroheptameric ring in the constitution of α, β, β, α. The α subunits govern the substrate entry while the β subunits fulfil hydrolytic function[19,23,24,35].

The 19S regulatory particles are responsible for the recognition and preparation of a substrate. The particles are composed of "base", further formed by AAA+-ATPase (RPT) and non-ATPase (RPN) subunits, and "lid", consisting of nine RPN subunits. Specific subunits are required for Ub-binding, Ub-removal, 20S opening, substrate binding, substrate unfolding, and substrate translocation[19,23,24].

The canonical Ub chain linked with lysins at position 48 (K48) is the principal signal for proteasome to degrade protein[17,18,36]. A shuttle protein and a subunit of the 19S proteasome then recognise the ubiquitinated substrate. The recognition activates DUB enzymes associated with the proteasome. DUBs cut part of the Ub chain, which leads to the recycling of Ubs. 19S particle directs the substrate to the 20S core particle while the substrate is unfolded and deubiquitinated. When the α subunit opens, the substrate is cleaved by β subunits[19,23,24,36].

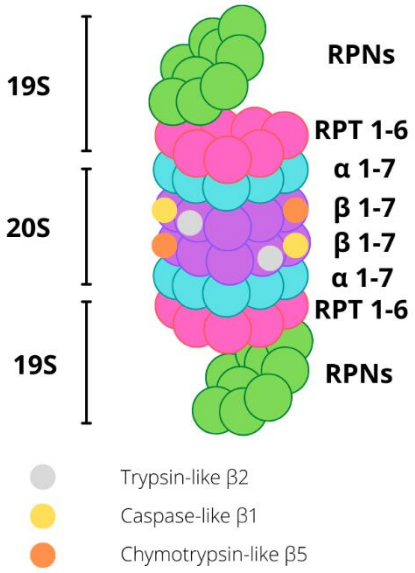

Fig. 2.: The 26S proteasome is assembled from the 20S core particle and the 19S regulatory particles. The core particle is further composed of α and β subunits. In addition, specific subunits fulfil hydrolytic activity. Regulatory particles consist of ATPase (RPT) and non-ATPase (RPN) subunits.
Abbreviation: RPT – Regulatory particle AAA+ ATPase, RPN – Regulatory particle non-ATPase

1.2. Protein quality control

During cell life, a large amount of proteins is synthesised and degraded. Apart from regular protein turnover, the cells struggle with misfolded proteins, mutant proteins, proteins erroneously located and faulty un/assembled multi-complex subunits. The cells develop several protein quality control mechanisms (PQC) to balance normal cell function and maintain protein homeostasis. PQC recognises aberrant proteins and promotes their refolding, sequestration, or degradation. A broad family of molecular chaperones orchestrate the control processes[1,14,15,37–39].

Several conditions may cause the overload of aberrant proteins resulting in proteotoxic stress, including ageing, DNA mutations, and exposure to exogenous factors (high temperature, drugs/chemicals, infections, nutrition insufficiency). Accumulation or production of excessive aberrant proteins is associated with various human diseases such as cancer, neurodegenerative diseases, and cardiovascular disorders[40,41]. In addition, induced proteotoxic stress is one of the strategies to treat cancer[26–29].

Molecular chaperones are responsible for protein folding, refolding, translocation, sequestration, and degradation assignment. Those linked to proteotoxic stress are heat shock proteins (HSPs) classified according to their molecular weight (small HSPs, HSP60, HSP70, HSP90, HSP100) and subdivided into specific isoforms. The isoforms play various roles. Additionally, they may be tissue-specific, subcellular-specific, constitutively expressed, or inducibly expressed[14,37].

The so-called unfolded protein response (UPR), which collaborates with the UPS and the autophagy-lysosome system, is an essential part of PQC. Synthesised proteins translocate from cytosol to the endoplasmic reticulum (ER), where chaperone-mediated folding occurs. Under an overload of unfolded proteins, ER stress response is induced involving three primary adaptive responses[1,38,39,42]:

1) The first response is accompanied by decreased mRNA translation through Protein Kinase RNA-Like ER Kinase (PERK)-mediated phosphorylation of eukaryotic initiation factor 2 subunit-α (eIF2α). The event has the effect of preventing newly synthesised proteins from occupying the ER. The exception has several mRNAs recognised by eIF2α, including Activating transcription factor 4 (ATF4), which further induces transcription of UPR-associated genes. The products of genes are involved in redox homeostasis, autophagy, apoptosis, amino acid metabolism, and protein folding.

2) The second pathway begins with the activation of a type 1 ER transmembrane protein kinase/endoribonuclease (IRE1α), which cleaves X-box-binding protein 1 (XBP1). XBP1 induces transcription of UPR-associated genes involved in inflammation response, ER/Golgi biogenesis, and ER protein translocation, folding, secretion and degradation. IRE1α also cleaves other RNAs (micro/mRNAs), resulting in the degradation of mRNAs via IRE1-dependent decay leading to a lowering of the mRNA pool. Additionally, IRE1α may bind some stress-induced proteins and activates inflammation, autophagy and stress response pathways.

3) ATF6 is activated by protease-mediated cleavage to begin the third pathway. Emerged fragment, referred to as ATF6p50, translocates to the nucleus and triggers transcription of UPR-associated genes. The genes are involved in ER/Golgi biogenesis, protein folding, secretion, and degradation.

XBP1 (2) or ATF6 (3) induce ER-associated protein degradation (ERAD), degrading unfolded proteins in ER. Alternatively, ATF4-mediated autophagy degrades the proteins. VCP/p97 conducts the transport of ubiquitinated proteins from ER to the proteasome, which enables the ERAD process[1,38,39,42].

When the cell does not manage the overload of unfolded protein, the proapoptotic response is activated. The proapoptotic signals come from PERK and IRE1α involving a cascade of events ending in apoptosis[1,38,39,42].

1.3. VCP/p97 complex

VCP/p97 AAA+ ATPase belong to the multifunctional proteins which fulfil their tasks by bound cofactors. It plays a crucial role in protein maintenance and segregation from cellular structures and protein complexes. VCP/p97 also contributes to protein degradation, associates with protein aggregates, regulates the cell cycle, DNA damage response, DNA replication, edits membranes, and regulates autophagy function and function of lysosomes[43–46].

VCP/p97 predominantly localises to the ER. Structurally, the protein consists of D1 and D2 ATPase domains. The two domains form homohexamer, which arises from two attached rings. While D2 is only responsible for ATPase hydrolysis, the D1 unit is in charge of the complex assembly with extra ATPase activity. In addition, the N-terminal domain required for substrate recognition and the C-terminal domain needed for nuclear localisation are additional functional structures[43–46].

Heterodimer UFD1-NPL4 is one of the cofactors that bind to VCP/p97. The function of the heterodimer is the recognition of ubiquitinated proteins. Thus, the segregation of ubiquitinated protein from cellular structure and protein complexes predetermined for degradation is the principal role of the whole VCP/p97-UFD1-NPL4 complex (Fig. 3). The complex has a different role in DNA replication and regulation of the cell cycle. Generally, these functions contribute to the proteome and genome stability[43,44,46].

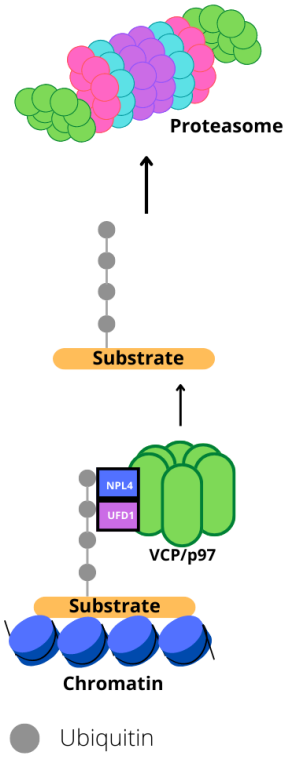

Fig. 3.: The VCP/p97-UFD1-NPL4 complex recognises polyubiquitinated substrate, a component of another structure (in this figure: chromatin). Complex segregates substrate from a structure. Proteasome subsequently degrades the substrate.

Abbreviation: NPL4 – Nuclear protein localisation protein 4, VCP/p97 – Valosin-containing protein, UFD1 – ubiquitin recognition factor in ER-associated degradation protein 1

1.4. Ubiquitin-proteasome system (UPS) and cancer therapy

The general phenotype of cancer cells is characterised by upregulated proteotoxic stress. The stress arises from increased protein synthesis and synthesis of defective proteins highly occupying UPS. Additionally, UPS was shown to have a role in tumour metabolism, immunological modulation and stemness maintenance. This UPS dependence provides potential targets for cancer treatment of various cancer types. Many selective molecules were developed, directly targeting the proteasome subunits or upstream processes, including (de)ubiquitinating enzymes, PQC, and protein segregation. Some of these molecules,

such as bortezomib, have already been FDA-approved for clinical use in oncological treatment[1,8,26–29].

Bortezomib is a reversible inhibitor of the 20S proteasomal β5 subunit leading to the inhibition of its chymotrypsin-like activity. It obtained its initial FDA approval in 2003 for refractory multiple myeloma (MM), and thus the drug was the first approved compound targeting UPS. Bortezomib applications further and quickly expanded[2,27,28]. Based on high toxicity

and cancer resistance arising from the Bortezomib treatment, another proteasome inhibitor was developed called Carfilzomib. In contrast to Bortezomib, Carfilzomib forms an irreversible bond with the β5 subunit of 20S proteasome. The drug was FDA-approved in 2012 for patients with MM, including those who underwent Bortezomib therapy. Even though Carfilzomib was less toxic and initially efficient in Bortezomib resistance patients, the drug was poorly soluble in water forcing the use of highly concentrated solvents and inducing additional resistance[3,27,28]. Ixazomib, FDA approved in 2015 as the first orally administrated proteasomal inhibitor, provided the solution to these limitations. As with Bortezomib, the Ixazomib mechanism was explained by a reversible bond to the β5 subunit of 20S proteasome[4,27,28].

Besides the approved drugs mentioned above, many other UPS interferers are under clinical trials, including disulfiram: NCT04521335[5], NCT03323346[6], NCT03950830[7]. DSF is a member of medications that have historically been used to treat a different condition, in this case, alcoholism. The drug is currently a hot candidate for repurposing in cancer therapy. The human body metabolises the DSF molecule into many compounds, including CuET (Fig. 4), which has previously been demonstrated as one of the causes of its anticancer action. Mechanistically, CuET anticancer activity involves targeting the NPL4 protein, a critical VCP/p97-complex cofactor. NPL4 is under the act of CuET aggregated, which results in proteotoxic stress arising from the aggregates' presence and the elimination of a critical link in the UPS pathways[8].

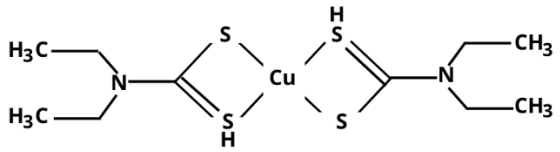

Fig. 4.: Molecule of bis-diethyldithiocarbamate-copper complex (CuET)

1.5. History of usage of *Cannabis* sp.

Cannabis sp. has a very long history that dates back more than ten thousand years to Asia, according to archaeobotanical records[47]. Over time, cannabis has gained a variety of uses as a fibre, culinary ingredient, medication, and spiritual substance[48,49]. European scientists began testing cannabis for medicinal applications in the 19th century, which sparked public and scholarly interest[49,50]. As a result, several companies started manufacturing cannabis products later prescribed for conditions like pain, inflammation, swelling, spasms, migraines, restlessness, and insomnia. Concerns about the recreational misuse of plants emerged in the 20th century, and as a result, the use of plants started to be regulated[49]. In the United States, restrictions were followed by establishing a high tax, even for industrial or medical use[49,51]. The plant has since been included on the list of addictive drugs[49].

Roger Adams began studying the chemical composition of cannabis in 1940. In a short time, he extracted and described CBD, synthesised cannabinol (CBN) and elucidated its molecular formula, and identified delta-9-tetrahydrocannabinol (Δ^9-THC) as a psychotropic component of cannabis. Regardless of its controversy, cannabis has come to the spotlight again after the separate discovery of the chemical structure of Δ^9-THC and CBD in the early 1940s by Gaoni and Mechoulan[49,52,53]. The discoveries sparked a fresh surge of interest in cannabis, particularly in connection with the endocannabinoid signaling system[49,54]. In recent years, numerous nations have authorised cannabis or its components again as medication, dietary supplement, or cosmetic, restoring a new wave of scientific interest[49].

1.6. Social and economic perspective of Cannabis

Cannabis, also known as marijuana, is a controversial drug used worldwide. Users of cannabis can be categorised into three categories based on whether they use it for recreational, therapeutic, or complementary purposes. Cannabis products are sold as purified cannabinoid-based products or plant parts[12]. The number of states that have legalised marijuana has increased in recent decades. The global market with marijuana in 2021 was valued at $25.7 billion, and the market for CBD alone was valued at $15.6 billion. Values of $148.9 billion and $59.43 billion are predicted for 2031 and 2030, respectively[55,56].

Nowadays, CBD products are easily accessible in internet shops, brick-and-mortar stores, and even in vending machines (Fig. 5) in shopping malls and gas stations in many countries worldwide. Moreover, CBD products are available as dried herbs, in foods, drinks, cosmetics, various pills, and extracts[12].

Fig. 5.: Vending machine offering CBD products in Olomouc, Czech Rep. (photograph taken in February 2023).

Besides the legal marijuana-based medications described in chapter 1.10, over-the-counter products are off-label and often lack CBD-content verification. These issues and the growing non-scientific public interest underline the need for a more profound examination of potential side effects, drug-drug interactions, and impacts on long-term users.

1.7. Chemistry behind cannabinoids

Cannabis sativa L., a member of the *Cannabaceae* plant family, can be classified into subspecies, the most well-known of which are indica, sativa, and ruderalis[57]. More than 500 compounds representing 18 chemical classes, including terpenes, flavonoids, non-cannabinoid phenols, and cannabinoids, were found in these plants[58–60].

Cannabinoids are characterised by C21 terpenophenolic backbone and could be divided into 11 subgroups[58,60]:

- Cannabidiol
- (-)-Δ^9-trans-tetrahydrocannabinol
- (-)-Δ^8-trans-tetrahydrocannabinol
- Cannabichromene
- Cannabigerol
- Cannabinol
- Cannabinodiol
- Cannabitriol
- Cannabielsoin
- Cannabicyclol
- Miscellaneous-type cannabinoids

Most cannabinoids are found in plants as carboxylic acids. Decarboxylation originates from heat (98–200 °C) and produces active forms[59].

From a medical standpoint, phytocannabinoids are the most examined plant's chemical class. For medical use, cannabis is legal in many countries, including the Czech Republic, Canada, Germany, Italy, the Netherlands, and almost half of the US states. It was

accepted despite potential adverse events affecting the cardiovascular, reproductive, respiratory, and central nervous systems and the risk for addiction development[61,62].

The most abundant cannabinoids in Marijuana are Δ^9-THC and CBD, both of which have significant medical potential. However, Δ^9-THC is problematic due to its psychoactive properties, and that is why non-psychoactive CBD started drawing more attention in the last decade.

The biological effects of cannabinoids are reported in numerous studies (reviewed in Morales et al. (2017)[13]). Interestingly, publications often came to opposing findings, mainly explained as effects of various models, concentrations, and experimental settings. Also, high non-physiological drug concentrations commonly used in research with reporting impact on multiple cellular targets are unachievable in the human body, further complicating the unambiguous translation of results. There is also an increasing number of various cannabis products, overwhelming their proper study. Furthermore, only a few cannabis products are standardised, and the chemical content may differ depending on plant variety, cultivation conditions, and manufacturing factors, complicating comparison of individual studies. Additionally, results may vary from patient to patient and form of administration, predominantly oral or through inhalation[59,63].

Nevertheless, there is no doubt that Marijuana and its derivatives have a significant potential for medical application. Unfortunately, due to scientific clutter, many people are encouraged to use marijuana and other products for situations where the benefits are questionable and potentially harmful[62].

1.8. Pharmacokinetics of cannabinoids

Due to the high complexity of cannabis's elemental composition, which varies depending on the parameters described above, detailed pharmacokinetics studies are limited. Moreover, one substance's altered concentration can quickly affect others' kinetics[59,63].

Generally, cannabinoids distribute to the adipose tissue and well-vascularised organs (brain, heart, lung, and liver)[59,63–67]. Eliminations of cannabinoids take from a few minutes (initial half-life) to days (long terminal elimination half-life)[59,63,68]. The general half-life for

orally administrated CBD and Δ^9-THC is 24 hours to 5 days for intravenously, smoked, or chronic oral administrated CBD. Cannabinoids are metabolised by many biotransformation enzymes, mainly responsible for decarboxylation, oxidation, and conjugation[57,59,63].

Essential parts of cannabinoid metabolism are cytochromes P450. Especially CYP2C9, CYP2C19, and CYP3A4 for Δ^9-THC, CBD, and CBN are well-studied[59,63,69–72]. Additionally, glucuronidation from phase II of biotransformation is fundamental for eliminating cannabis metabolites[59,63,73]. Finally, terminal metabolic waste products are eliminated by faeces and urine[63,74–76].

The peak plasma concentration of inhaled cannabinoids (Δ^9-THC, CBD) is reached within the first few minutes, and their systemic bioavailability is typically between 10-35 % (Δ^9-THC) and 31 % (CBD) of the received dose. However, orally administrated cannabinoids (Δ^9-THC, CBD) have only about 6 % bioavailability. Low bioavailability may result from their lipophilic nature[63,74,77,78]. Guy and Flint (2004)[79], for example, compared the pharmacokinetics of Δ^9-THC, CBD, and Δ^9-THC:CBD in human subjects after a single oral dose of 20 mg of each substance. Cannabinoids CBD and Δ^9-THC had maximal plasma peak concentration after 32-100 minutes with 2,05-12,46 ng/ml concentrations and half-life from 47 to 144 min[79]. Additionally, 700 mg of CBD was given orally to patients with Huntington's disease every day for six weeks while their plasma concentrations were monitored[80]. After six weeks, the CBD plasma level was 5,9-11,2 ng/ml. A week after the termination of the drug administration, the level of CBD was 1,5 ng/ml with an estimated elimination time of 2-5 days[80].

1.9. Pharmacodynamics of cannabinoids

CBD is the subject of most pharmacodynamics research within cannabinoids due to its high medical potential, abundance in the plant, and non-psychoactive properties. Some cellular targets were also uncovered for Δ^9-THC, Δ^8-THC, CBN, cannabigerol, cannabichromene, Δ^9-tetrahydrocannabivarin, and cannabidivarin[13,49,81,82], see Tab. 1.

Tab. 1.: Activity of cannabinoids against their targets (Table adjusted and taken from Morales et al. (2017)[13].

	Δ^9-THC	**Δ^8-THC**	**CBN**	**CBD**	**CBG**	**CBC**	**Δ^9-THCV**	**CBDV**
CB1	Partial agonist	Partial agonist	Agonist	Antagonist or negative allosteric modulator	Partial agonist	Agonist	Antagonist	NR
CB2	Partial agonist	Partial agonist	Agonist or inverse agonist	Antagonist	Partial agonist	Agonist	Partial agonist	NR
AEA uptake			-	Inhibitor	Inhibitor	Inhibitor	-	-
GPR55	Agonist, LPI inhibitor, or NR	-	-	Antagonist	LPI inhibitor		Partial agonist, LPI inhibitor	LPI inhibitor
GPR18	Agonist	-	-	Antagonist	-	-	-	-
5-HT1A	-	-	-	Agonist	Antagonist		Agonist	
5-HT2A	-	-	-	Partial agonist	-	-	-	-
5-HT3A	Antagonist	-	-	Antagonist	-	-	-	-
μ- and δ-OPR	Allosteric modulator	-	-	Allosteric modulator	-	-	-	-
PPARγ	Agonist	-	-	Agonist	-	-	-	-
GlyR α1	Positive allosteric modulator	-	-	Positive allosteric modulator	-	-	-	-
GlyR α3	Positive allosteric modulator	-	-	Positive allosteric modulator	-	-	-	-
TRPV1, 2, 3, 4	TRPV 2, 3, 4 agonist	-	-	TRPV 1, 2, 3 agonist	TRPV1, 2 agonist	TRPV3, 4 agonist	TRPV2 agonist	TRPV 1, 2, 3 agonist
TRPA1	Agonist	-	Agonist	Agonist	Agonist	Agonist	Agonist	Agonist
TRPM8	Antagonist	-	Antagonist	-	Antagonist	Antagonist	Antagonist	-
α2-AR	-	-	-	-	Agonist	-	-	-

Abbreviation: Δ^8-THC – delta-8-tertrahydrocannabinol, Δ^9-THC – delta-9-tertrahydrocannabinol, Δ^9-THCV – delta-9- tetrahydrocannabivarin, CBC – cannabichromene, CBD – cannabidiol, CBDV – cannabidivarin, CBG – cannabigerol, CBN – cannabinol, NR – no response

All the cannabinoids were first studied concerning the cannabinoid receptors 1 and 2 (CB1, CB2). CB1 and CB2 are G-protein coupled receptors belonging to the endocannabinoid system. Its well-known endogenous ligands are anandamide[54,83] and 2-arachidonoylglycerol[54,84,85]. CB1 is predominantly expressed in the brain, while CB2 is mainly expressed in immune cells[86,87]. Its activation leads to many physiological processes according to the cell type. Generally, the receptors contribute to the following[87]:

- Neuroprotection
- Behavioural modulation
- Appetite modulation
- Energy metabolism
- Immunity
- Nociception
- Cell growth
- Cell proliferation

The broad field of affected processes gives the excellent potential for the treatment of a wide range of disorders, diseases, and their symptoms which are in a well-arranged way discussed in Stasiulewicz et al. (2020)[87].

Except for CB1 and CB2, cannabinoids also interact and modulate[13]:

- G protein-coupled receptor 55 (GPR55) and 18 (GPR18)
- 5-hydroxytryptamine receptor 1A (5-HT1A), 2A (5-HT2A), and 3A (5-HT3A)
- mu- and delta-opioid receptor (μ- and δ-OPR)
- Peroxisome proliferator-activated receptor gamma (PPARγ)
- Glycine receptor (GlyR) subunits α1 and α3
- Transient receptor potential (TRP) channels
- alpha-2 adrenergic receptor (α2-AR), all characterised below.

GPR55 and GPR18 were suggested as additional cannabinoid receptors[88,89]. GPR55 role stands in the modulation of energy homeostasis[90], bone physiology[91], may contribute to malignant cell growth[92], modulates innate immunity[93] and central nervous system (CNS)[94,95].

In the case of CNS, the receptor impacts anxiety and motor coordination[94,95]. In contrast, GPR18 may play a role in metabolism[96] and immune modulation[97], including the modulation of intestinal intraepithelial lymphocytes[98].

5-HT receptors localise to the CNS and peripheral nervous system, which have inhibitory or excitatory functions by modulating the release of various neurotransmitters or hormones[99,100]. They include presynaptic autoreceptors and postsynaptic heteroreceptors. These divide into seven classes (5-HT1 – 5-HT7) comprising 14 subtypes of receptors[101], from which three subtypes are affected by cannabinoids (5-HT1A, 5-HT2A, and 5-HT3).

5-HT1A is vital in the modulation of mood, emotions[102–104], and pain[105]. 5-HT2A has a role in pain[106,107], memory, and cognition[108,109]. Receptor 5-HT3 influences anxiety[110], cognition[111], pain sensation[112], and memory[113].

PPARs are nuclear hormone receptors comprising three isotypes[114,115]: PPARα, PPARβ, and PPARγ, with γ isotype relevant within cannabinoids. PPARγ modulates fatty acid storage[116,117], insulin sensitivity[118], and neuroprotection[119]. Furthermore, PPARγ shows proapoptotic[120] and antiproliferative properties[121].

GlyR is a ligand-gated ion channel localised to the CNS that may comprise four different α subunits and one β subunit[122]. According to the homo- or heteropentameric configuration, the receptor has different pharmacological effects[123]. Briefly, it has a function in neurodevelopment[124], inflammatory pain sensation[125], motoric control[126], sensory function, and respiration[123].

TRP channels are involved in various cellular processes by modulation of ion transport. Those, which are influenced by cannabinoids, have the following roles:

- Transient receptor potential vanilloid 1 (TRPV1), together with Transient receptor potential ankyrin 1 (TRPA1), is concentrated and plays a role in peripheral sensory neurons responsible for the perception of temperature[127,128], pain[129,130], and itch[131,132]. Moreover, the TRPs modulate inflammatory and dermatitis responses[133,134].
- TRPV2 potentially mediates and is induced by inflammation[135,136] and responses to thermal stimulation[137].

- TRPV3 is associated with the sensation of pain[138], itch[139], and temperature[140,141].
- TRPV4 responses to pain[142], mechanical stimuli[143], and temperature[144]. Additionally, this one is responsible for osmotic balancing[145] and the sensation of osmotic changes[146].
- Finally, the Transient receptor potential melastatin 8 (TRPM8) acts as another thermal sensor[147].

AR family of receptors comprising α1, α2, β1, β2, and β3 subtypes play a wide variety of roles, such as in metabolic regulation, cardiac function, muscle contraction, blood pressure, and neural modulation[148–150]. Namely, the α2-AR receptor involved in pain sensation, induction of sedation[151], insulin release[152], and norepinephrine release[153] is influenced by cannabinoids

The opioid receptors are also affected by cannabinoids involving μ- and δ- and κ-OPR receptors, all having analgesic functions[154,155] and an effect on behaviour[156] and locomotion[157].

1.10. Cannabis medicinal applications

Numerous medical conditions have been suggested as therapeutically targetable by cannabis and cannabinoids. Multiple sclerosis offers an example. It is an inflammatory and demyelinating neurodegenerative disease, which is nowadays incurable. Therefore, the treatment focuses on slowing down and symptoms management[158]. In this case, cannabis formulation (Sativex, Δ^9-THC:CBD, 1:1) is approved in several countries for treating specific symptoms, potentially leading to a better quality of life[159,160].

The effects of cannabis and cannabinoids on movement disorders such as Tourette syndrome, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and cervical dystonia have also been examined. For example, patients with Tourette syndrome, a neurodevelopmental disease characterised by tics and obsessive-compulsive behaviours, were investigated when given Δ^9-THC. It was determined that Δ^9-THC might help reduce symptoms[161]. Parkinson's disease belongs to the neurodegenerative disorders characterised

by tremors and bradykinesia. Cannabinoids or smoked cannabis were tested in several studies evaluating symptoms with conflicting results[162,163].

The symptoms of neurodegenerative Huntington's disease include chorea, dystonia, and impairment of motor ability, cognition, and behaviour. In a research of the symptoms, cannabidiol and the synthetic cannabinoid Nabilone were tried, and the results showed weak or no effect[80,164].

The neurodegenerative condition known as amyotrophic lateral sclerosis is characterised by progressive paralysis and cramping. A study on the effects of Δ^9-THC on crumps found no difference between the Δ^9-THC group and the placebo group[165]. Cervical dystonia is a neurological disorder characterised by focal dystonia. Dronabinol, a synthetic cannabinoid, was examined but had no impact on disease symptoms[166].

In several studies, cannabis and its products were tested against psychiatric conditions such as anxiety, sleeping disorders, and psychosis. For example, CBD administration improved anxiety[167]. Sleeping disorders were tested with Nabilone and Dronabinol with the probably beneficial result[168,169]. Psychosis was studied with CBD, and a potential antipsychotic effect was reported[170].

Epilepsy is a neurological disorder associated with seizures. In this case, CBD-based medication Epidiolex/Epidyolex received FDA and European Commission approval to treat several forms of the condition[171,172]. Nabilone effect, for instance, was evaluated against agitation in the context of Alzheimer disease and presumably had a 'probably beneficial' result[173].

The pain in combination with cannabis and cannabinoids is highly studied. However, the conclusions are conflicting. The studies could be categorised by the following types of pain: acute, chronic, disease-associated, and neuropathic pain. Montero-Oleas et al. (2020)[62] summarise systematic reviews evaluating studies and trials.

Together with cannabinoids and cannabis, cancer is another broad subject that has received extensive research. Studies were conducted to find therapeutic effects against chemotherapy-induced adverse events and cancer-associated symptoms. For example, Nabilone and Dronabinol are approved cannabinoid-based drugs for treating nausea and

vomiting induced by anticancer drugs[174]. In the case of cancer-related symptoms, including pain, the results vary from a 'harmful' to a 'beneficial' conclusion, see Montero-Oleas et al. (2020)[62].

Rheumatic conditions are characterised by chronic pain and gather several diseases. For example, rheumatoid arthritis is an autoimmune disease affecting mainly joints. Sativex was tested against disease-associated symptoms with 'potentially beneficial' effects at attenuation of pain[175].

The final example is fibromyalgia, a syndrome characterised by musculoskeletal pain. Nabilone was tested in fibromyalgia patients, but the studies had conflicting results[168,176].

Most studies' findings were inconsistent, with the commonest conclusions being 'probably beneficial' or 'unclear'. Methodological limitations are the primary explanation[62].

Many other conditions tested in combination with cannabis and its products were researched; for a summary of systematic reviews, see Montero-Oleas et al. (2020)[62].

1.11. Cannabidiol

A cannabinoid family is a group of phytoconstituents found in the cannabis plant, and CBD is a member of this group. Due to its higher proportional presence in plants, lack of psychotropic effects, and contentious traits mostly linked to 9-THC, CBD offers a wide range of research opportunities. Additionally, CBD is typically considered a safe substance with mild adverse effects[177].

1.11.1. Structure of cannabidiol

The biosynthetic pathway starts with a C18 fatty acid cleaved to hexanoic acid. This C6 is further converted to the hexanoyl-CoA and elongated to C8 tetraketide-CoA. Next, C8 is cyclised, forming olivetolic acid. In this step, C10 isoprenoid geranyl-PP, coming from the methylerythritol 4-phosphate pathway, enters the reaction. It prenylates olivetolic acid and thus forms cannabigerolic acid. The endpoint product, cannabidiolic acid (CBDA), is formed within the last oxidative cyclisation reaction. Decarboxylation to a neutral form occurs with

drying and heating without enzymatic contribution. Finally, a 21-carbon terpenophenolic compound is created, called (-)cannabidiol[178] (Fig. 6).

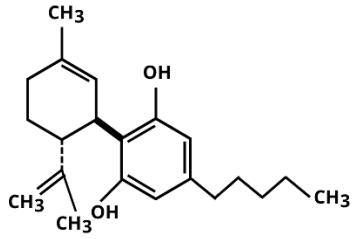

Fig. 6.: -(-)Cannabidiol molecule

1.11.2. Medical targets of cannabidiol

CBD has been extensively investigated over the past few decades due to its wide range of cellular targets and non-psychoactive properties. Among medical properties attributed to CBD belong:

- Anti-inflammatory[179]
- Antioxidant[180]
- Anxiolytic[181]
- Antidepressive[181]
- Antipsychotic[181]
- Anticonvulsant[182]
- Analgesic[183]

These properties candidate CBD for the treatment of the following pathological conditions:

- Diabetes[184]
- Cancer[185]
- Arthritis[186]
- Anxiety[187]
- Psychosis[187]
- Epilepsy[187]

- Cardiovascular diseases[188]
- Neurodegenerative diseases[189,190]
- Skin diseases[191]

1.11.2.1. Cannabidiol molecular targets - the transcriptional level

CBD transcriptionally alters cells practically in all aspects. For example, the following groups of transcripts were affected by CBD in BV-2 microglial cells[192,193]:

- Stress response
- Transcription regulation
- Metabolism
- Membrane transport
- Membrane secretion
- Phosphatases
- Adhesion
- Migration
- Motility
- Morphogenesis
- Apoptosis
- Cell cycle
- Proliferation
- G-protein coupled receptors
- Kinases
- Regulation of translation
- Inflammatory chemokines
- Inflammatory receptors
- Host defence
- Adaptive response

Juknat et al. (2012)[193] also elaborate on the alterations emphasising zinc (Zn^{+}) homeostasis, including metallothioneins (MTs). MTs will be discussed in more detail in chapter 1.13.1.

1.11.2.2. Cannabidiol molecular targets - protein level

The endocannabinoid system belongs among the most studied cellular targets comprising CB1 predominantly distributed to the brain and CNS, CB2 mainly localised to the peripheral nervous system and immune system, and relatively new classified GPR55[88,194,195]. CBD acts as an inverse agonist for the CB2 receptor[196,197], an antagonist of GPR55 receptors[88] and is reported to be a negative allosteric modulator or inverse agonist of CB1[196–198]. Based on the opposing effects of CBD and Δ^9-THC on the CB1 receptor, CBD might attenuate Δ^9-THC mediated adverse effects[198,199]. Studies for treating bowel disorders, neurological diseases, and inflammatory skin diseases reveal that CBD's anti-inflammatory activities may be partially explained by its inverse agonistic activity at CB2[197,200,201]. CBD's antiseizure effect was shown to be partially mediated by its antagonistic effect on GPR55[202]. Next, CBD was shown to decrease the efficacy of 2-AG signalling[198] and to inhibit Fatty acid amide hydrolase (FAAH). Notable, FAAH inhibition causes an increased pool of anandamide in cells[203]. Contrarily, Massi, et al. (2008)[204] reported CBD-mediated induction of FAAH activity.

TRP channels modulating intracellular calcium (Ca^{2+}) and controlling temperature, pain, osmolar and mechanic perceptions are highly studied concerning CBD. CBD is an agonist of TRPV1 and TRPV2 channels and causes an increase in intracellular Ca^{2+} levels[135,205,206]. Several CBD-mediated effects are attributed to the modulation of TRPV1. For example, CBD-mediated anticonvulsant effects are linked with TRPV1 (and CB1 and CB2 receptors)[207]. Furthermore, CBD reduced cocaine reward behaviour, reversed by the administration of TRPV1 (and CB2 and 5-HT1A) inhibitors[208]. Additionally, CBD has antihyperalgesic properties driven by TRPV1[209]. CBD was further shown to modulate TRPA1 and adenosine A1 resulting in antinociceptive properties[210].

PPARγ plays a role in glucose and lipid metabolism and insulin signalling. Notably, the highest expression occurs in adipocytes, where PPARγ controls adipocytokines secretion and lipid loads[211]. CBD is reported to be an agonist of this receptor[212], which suggests a positive effect on Type 2 Diabetes. However, this was not proven in a clinical trial (NCT01217112[213])[214]. PPARγ was further associated with Alzheimer's disease, and CBD was proposed to be effective in attenuating Alzheimer's disease manifestation[215].

Furthermore, CBD is an agonist of serotonin receptor 5-HT1A[216]. The family of serotonin receptors plays roles in diverse general processes through neuronal excitability and neurotransmitter releases, such as development, behaviour, sensation, and endocrine function[217]. CBD was shown to be an agonist of 5-HT1A[216]. In association with 5-HT1A, CBD was studied as a potential anti-anxiety compound[218], substance attenuating mechanical allodynia[219], and in cooperation with CB2, substance decreasing neuronal inflammation[201].

Among reported targets, few cellular transporters have been identified, including ATP binding cassette subfamily G member 2 (ABCG2)[220] and p-glycoprotein (p-gp)[221], both of which CBD inhibits, reducing drug efflux. The information is relevant for drug contraindications leading to increased drug toxicity. Contrarily, Epidiolex prescribing information does not anticipate interactions with p-gp transporters but cautions at the possibility of interference with ABCG2 and ABCB11 through 7-COOH-CBD, a metabolite of CBD[222]. This topic will be more examined in chapter 1.12.1.

A subset of the cytochrome P450 (CYP450) biotransformation enzymes, including CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5, were found to be responsible for CBD's metabolism. The two main enzymes that appear to be in charge of metabolism are CYP2C19 and CYP3A4[69]. These two enzymes[223] together with UDP-Glucuronosyltransferases (UGTs) UGT1A9, UGT2B7[224], and CYP450s: CYP1A2, CYP2B6, CYP2C8, and CYP2C9 are also highlighted in the prescribing information for Epidiolex, which warns against potential interactions with other medications that are metabolised by the CYP450s[222]. The drug-drug interactions based on biotransformation enzymes are described in more depth by Brown & Winterstein (2019)[12].

Along with the previously mentioned CBD targets and pathological conditions, others are suggested, including (Tab. 2):

Tab 2.: List of conditions potentially treatable by CBD

Target	Pathological condition	Reference
Adenosine A1A	Antiarrhythmic effect	Gonca & Darici (2015)[225]
Adenosine A2A	Anti-inflammatory effect	Carrier et al. (2006)[226]
μ-OPR and δ-OPR	Drug-seeking behaviour	Kathmann et al. (2006)[227] Viudez-Martínez et al. (2018)[228]
GlyR subunits α1, α3, and β2	Anti-inflammatory, antinociceptive, and neuroprotective properties	Ahrens et al. (2009)[229] Xiong et al. (2012)[230]
Gamma-aminobutyric acid type A (GABA-A) receptor	Anticonvulsant and anxiolytic effects	Bakas et al. (2017)[231]
TRPV3	Inflammation	De Petrocellis et al. (2012)[232]
Dopamine D2	Antipsychotic properties and antiepileptic properties	Seeman (2016)[233] Ghovanloo et al. (2018)[234]
Calcium channels	Antiarrhythmic and neuroprotective properties	Ali et al. (2015)[235] Ryan et al. (2009)[236]

1.12. Cannabidiol and cancer

Nowadays, cancer patients frequently utilize CBD or marijuana in general as a palliative care component or as a way to lessen the side effects of chemotherapy (nausea, vomiting, pain, loss of appetite)[237]. Additionally, CBD is being thoroughly researched as a potential alternative therapy for cancer in a variety of cancer models, including lung[238], glioma[239], leukaemia[240], melanoma[241], endometrial[242], breast[243], and colon cancer[244].

It has been demonstrated that CBD has anti-proliferative and pro-apoptotic capabilities and actions that limit cancer cell angiogenesis, migration, adhesion, and invasion[199,245,246].

1.12.1. Cannabidiol as a chemotherapy adjuvant

Over the past few years, CBD has undergone substantial research as a potential chemotherapy adjuvant. There are two methods for assessing CBD contribution:

1. The first method evaluates CBD as a substance reducing the harmful effects of chemotherapy. CBD attenuated oral mucositis in mice treated with 5-fluorouracil[247]. Furthermore, CBD decreased cisplatin-induced renal toxicity in mice[248] and, in low doses, also cisplatin-induced emesis in shrews[249,250]. In addition, CBD was reported to attenuate oxaliplatin-induced neuropathy pain in mice[251]. Likewise, CBD forestalls peripheral neuropathy in mice caused by paclitaxel[251–253]. Similarly, CBD attenuated doxorubicin-induced cardiac injury in rats and mice[254,255].
2. A second strategy assesses the prospective potentiation of chemotherapeutic effectiveness. Indeed, there are preclinical examples. For example, a mouse model with pancreatic ductal adenocarcinoma cotreated with CBD and gemcitabine outlived mice treated with gemcitabine alone[256]. Moreover, CBD was shown to increase the drug uptake of carmustine in glioblastoma cellular models[257,258] and overcome drug resistance of glioblastoma stem-like cells[259]. Additionally, CBD enhances the effectiveness of temozolomide in the glioblastoma cellular model[258,260,261] and mouse xenografts[261]. Likar et al. (2019)[262] found that patients who received radiation, temozolomide, and CBD treatment had unexpectedly extended lifetimes. However, preclinical mouse models involving glioblastoma xenografts did not confirm the increased efficacy of the combination[263]. CBD was also reported to potentiate cisplatin treatment[264], although this finding was not confirmed by another group using a different model[265]. The opposite result was obtained by Marzęda et al. (2022)[266], who showed antagonistic effects of CBD and cisplatin in melanoma cell lines. CBD has overcome resistance against oxaliplatin in mice xenografts and colorectal cancer cells[267]. Additionally, an antagonistic effect of CBD on carboplatin was reported[268]. CBD increased the toxicity of vinblastine due to a decrease in p-gp transporter efflux[269]. *In ovo* studies at chicken embryo chorioallantoic membrane revealed that combined CBD plus paclitaxel are more effective than either treatment alone[265,270]. The effect was further seen in another cell line[264], although Sainz-Cort et al. (2020)[271] did not confirm this in different cell lines. In prostate cancer models, CBD at lower concentrations enhanced the cytotoxic effects

of docetaxel[272]. Higher chemotherapy efficacy of vincristine after CBD was also suggested for ovarian cancer cell lines and canine neoplastic cell lines[273,274]. Additionally, CBD potentiated doxorubicin treatment by the increased influx and decreased efflux in:

- Colorectal adenocarcinoma cell line[221]
- Glioblastoma cell line[257]
- Hepatocellular carcinoma cell line[275]
- And other cell lines[264,276,277].

Bortezomib was more efficient in multiple myeloma cell lines in combination with CBD[278]. On top of that, CBD inhibited topotecan efflux and thus increased its toxicity in the mouse cell line overexpressing the ABCG2 transporter[220].

CBD, in combination with several chemotherapies, is also planned for several clinical trials (Tab. 3):

Tab 3.: Clinical trials of CBD in combination with chemotherapy.

5-Fluorouracil	Colon or rectal cancer	NCT03607643[279], status: Not yet recruiting
Gemcitabine	Pancreatic cancer	
Temozolomide	Glioblastoma multiforme	
Bortezomib	Multiple myeloma	
Oxaliplatin	Colon or rectal cancer	
	-	NCT04582591[280], status: Not yet recruiting
Paclitaxel	-	
Neurotoxic chemotherapy	Colorectal, breast, uterine, ovarian, pancreatic	NCT04398446[281], status: Not yet recruiting
Temozolomide	Glioblastoma multiforme	NCT03687034[282], status: Not yet recruiting

1.13. The link between cannabis and protein homeostasis

CBD's biological effects also include numerous targets associated with UPS and thus potentially affects proteostasis. For example, CBD modulates the transcription level of some ER-stress-responding proteins in microglial cells[192]. Nevertheless, predominant effects resulting in the protection of cellular proteins are indirect. It has frequently been demonstrated that CBD acts on anti-inflammatory, anti/pro-oxidant pathways, various channels, and receptors involved in neurodegeneration[180,190,283,284]. Growing evidence for the involvement of CBD in protein homeostasis results in clinical trials testing CBD against neurodegenerative and neurodevelopmental diseases(Tab. 4):

Tab 4.: List of clinical trials testing CBD in patients of neurodegenerative and neurodevelopmental diseases.

NCT03582137[285] Status: Completed	Parkinson's disease
NCT04436081[286] Status: Recruiting	Alzheimer's disease
NCT02544763[287,288] Status: Completed	Tuberous Sclerosis Complex
NCT02544750[289] Status: Completed	Tuberous Sclerosis Complex
NCT04485104[290] Status: Recruiting	Tuberous Sclerosis Complex

1.13.1. Metallothioneins

CBD positively regulates the metallothionein (MT) pathway[193]. MTs are a family of small proteins involved in cellular transport, storage, and elimination of biogenic or toxic metals[291–293] (Fig. 6). Although their primary purpose is to maintain the balance of Zn^{+} and copper (Cu^{2+}), their structure with high cysteine content allows binding of heavy metal cations (mainly divalent)[293], thus protecting the cellular proteins. MTs are also involved in oxidative stress response[294] and bind even non-metal drugs[295,296] serving as broad-spectrum detoxificators. They also play a role in controlling cell growth, proliferation, and carcinogenesis[291].

MTs divides into four classes: MT-1, MT-2, MT-3, and MT-4. MT-1 and -2 (or MT-2A) are inducible and expressed in many tissues, especially in the liver, pancreas, and kidneys[292,297,298]. MT-1 is further subdivided into functional genes *MT-1A, -1B, -1E, -1F, -1G, -1H, -1H like 1, -1M, -1X*, and pseudogenes *MT-1C, -1D, -1I, -1J, -1L, -1P1, -1P3*[297,299]. Even though certain MT-1 isoforms perform essentially the same function, they might be preferentially expressed in different tissues[291,292]. MT-3 is expressed in the brain and male reproductive system, while MT-4 is found in the skin and upper intestinal tract. Both classes (MT-3, MT-4) are expressed constitutively[292,297,298]. In contrast to MT-3, which is more important for neurodevelopment and brain protection[300,301]. MTs activity lies in the ability to chelate metals and thus protects, besides other things, cellular proteome against (in)direct damage caused by the harmful metals [291–293].

The primary transcriptional regulator for basal and inducible MT-1 and -2 is Metal-responsive element-binding transcription factor 1 (MTF1)[302]. MTF1 senses and binds excess of Zn^{+} in the cytoplasm and thus is activated to trigger MTs transcription. In addition, other metals activate MTF1 indirectly through an increase in free intracellular Zn^{+} released from Zn^{+}-saturated MTs[303–305].

MTs are suggested as prognostic markers for a variety of malignancies as well as chemotherapy responses, which is in-depth summarised in numerous reviews[291,292,297,298].

2. AIMS

1. Perform a screen for substances interfering with the biological activity of disulfiram's metabolite bis-diethyldithiocarbamate-copper complex (CuET)
2. Experimentally confirm and show the interfering potential of identified substance(s)
3. Uncover the mechanism of action of identified interfering substance(s)

3. MATERIALS AND METHODS

3.1. Cell lines

Human osteosarcoma cell lines U-2-OS (ATCC), U-2-OS ectopically expressing NPL4-GFP (Skrott et al., 2017), U-2-OS ectopically expressing MT-2A-GFP, human breast adenocarcinoma MDA-MB-231, and retinal pigment epithelia hTERT RPE-1 (ATCC) were used. Cells were cultured in DMEM media (Lonza) supplemented with 10% fetal bovine serum (Thermo Fischer Scientific) and 1% penicillin/streptomycin (Sigma-Aldrich).

3.2. High throughput screening for detection of CuET activity interferers

U-2-OS-NPL4-GFP cells were seeded in 384-well plates (Perkin Elmer, CellCarrier-UltraPlate) at a density of 1250 cells per well. The cells were pre-treated with library of 1282 compounds at 10μM concentration for 17 hours, followed by treatment with 0.2μM CuET for 3 hours. The cells were then pre-extracted using 0.2% Triton X-100 buffer containing 10μM Hoechst 33342. Next, the cells were washed by 1xPBS, and fixed by 1% formaldehyde for 10 minutes. The last step was washing with 1xPBS and adding 30 μl of 1xPBS to each well.

An automated microscopic platform was used to acquire each well (Yokogawa CV7000, 10 x air objective). Four positions per well were taken and analysed by Columbus (Perkin-Elmer). The Hoechst dye signal was used to identify nuclei. Finally, the intensity of the NPL4-GFP signal was analysed, and means were plotted to the dot plot.

3.3. Gene silencing and transient overexpression

Transfections were performed with siRNA against MTF1 (cat. n.: SR302991, OriGene), siRNA against MT-2A (cat. n.: SR302987, OriGene) expression plasmid containing Myc-DDK-tagged MT-2A (cat.n.: RC202748, OriGene) and expression plasmid with GFP-MT-2A (cat.n.: RG202748, OriGene). Gene silencing and transfection of plasmids

were performed using Lipofectamine RNAiMAX (cat. n.: 13778-075, Invitrogen) and Lipofectamine 2000 (cat. n.: 11668-027, Invitrogen), respectively. The protocol was followed according to the manufacturer's instructions. Cells were reseeded after 24 hours and treated after additional 48 hours.

3.4. Establishment of stable cell lines

Cells were transfected with an expression plasmid carrying TurboGFP-tagged MT-2A (cat. no: RG202748, OriGene). For the transfection, Lipofectamine 2000 (cat. no: 11668-027, Invitrogen) was used, and the protocol was followed according to the manufacturer's instructions. Next, the cells were cultivated in the presence of selective antibiotics (Geneticin, G418; Sigma, 400 $\mu g \cdot mL^{-1}$), which were changed every 2–3 days. After establishing new cell lines, originating from a single cell, clonal cell lines were produced.

3.5. 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay

The cells were seeded in a 96-well plate at a density of 5000 cells per well and treated as described in the figure legends. XTT assay (Applichem) was carried out as described in the manufacturer's instructions. In short, the XTT solution was applied to the media and incubated for 30-120 minutes. The dye intensity was measured at the 475nm wavelength using a spectrometer (TECAN, Infinite M200PRO). The results are presented as mean values and standard deviations from 3 independent experiments performed in 5 technical replicates.

3.6. Cell fractionation

Cells were seeded on a dish or plate and treated as described in figure legends. Next, the cells were washed with 1x PBS and incubated with lysis buffer (50 mM HEPES pH 7,4; 150 mM NaCl; 2 mM MgCl2; 10% glycerol; 0,5% Triton-X; protease inhibitor cocktail by Roche) for 10 minutes at 4 °C, gently agitated. Cells were harvested with the use of a scrapper and transferred into Eppendorf tubes. After that, the samples in tubes were incubated on ice for 10 minutes and then centrifuged for 10 minutes at 4°C at 20 000 x g. The supernatant was

transferred to the new Eppendorf tube, and both fractions (supernatant and pellet) were lysed by 2x LSB buffer. Prepared samples were analysed with the use of immunoblotting.

3.7. Immunoblotting

Cell lysates were separated by SDS-PAGE on a hand-cast (8%, 15%) gels or 4-15% Mini-PROTEAN TGXTM Precast Gel (BIO-RAD) gels and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% bovine milk dissolved in TBS-Tween 20 (0,1%) for 1 hour at room temperature. Next, the membrane was incubated with primary antibody overnight at 4°C, washed three times by TBS-Tween 20, and incubated with secondary antibody for 1 hour at room temperature. After washing steps, the secondary antibodies were visualized using Immobilon Forte Western HRP Substrate (cat. n.: WBLUF0500, Merck Millipore). Finally, images were acquired by the ChemiDoc imaging system (Bio-Rad).

3.8. Quantitative fluorescence microscopy

The cells were seeded on glass coverslips and treated as described in the figure legends. Next, the cells were pre-extracted for 1 minute with pre-extraction buffer (0,5% Triton X-100), washed with 1xPBS, and with 4% formaldehyde for 15 minutes at room temperature. Alternatively, the cells were fixed firstly in 4% formaldehyde for 15 minutes and then permeabilised with 0,5% Triton X-100 for 20 minutes. In both protocols, nuclei were stained with DAPI (1µg/ml) at room temperature for 5 minutes. Fluorescence microscopes were used to visualise and acquire samples (Zeiss LSM780 or Olympus IX81 ScanR automated microscope). ScanR Analysis software was used to perform quantitative analysis of microscopic data. STATISTICA 13 (TIBCO) was used to assess the data obtained and processed by ScanR.

3.9. Quantitative polymerase chain reaction

The qPCR was performed in a 96-well plate or 8-tube strip (Roche). LightCycler Nano (Roche) or LightCycler 480 Instrument II (Roche) were used for the reactions. Samples were run in the presence of a "gb SG PCR Master Mix" (cat.n.: 3005, Generi Biotech) or "Fast SYBR Green Master Mix" (cat.n.: 4385612, Applied Biosystems). The primers used were as follows:

- HSPA1A[306]:

  Forward 5′- GCCTTTCCAAGATTGCTGTT-3′

  Reverse 5′-TCAACATTGCAAACACAGGA-3′

- MT-1E:

  Forward 5′- GCCTGACTGCTTGTTCGTCT -3′

  Reverse 5′- AAGAGCAGTTGGGGTCCATT -3′

- MT-2A:

  Forward 5′- CCCGCTCCCAGATGTAAAGA-3′

  Reverse 5′- TAGCAAACGGTCACGGTCAG -3′

- GAPDH[307]:

  Forward 5′- AGCCACATCGCTCAGACAC -3′

  Reverse 5′- GCCCAATACGACCAAATCC -3′

  The delta-delta CT method was used to assess gene expression.

3.10. Measurement of CuET in culture medium and cells

A complete cell culture media (DMEM, 10% FBS, 1% penicillin/streptomycin) was incubated with CuET or CBD+CuET combination as described in the figure legend. The media was then vortexed and mixed with acetone in a 1:4 ratio. The mixture was centrifuged

at 18 000 x g for 2 minutes at 4°C. For measurement, the supernatant was put into glass HPLC vials. The CuET complex was measured using the HPLC-MS technique previously reported[8]. The calibration curve was used to calculate the quantification of the CuET complex.

Sub-confluent U-2-OS cells were treated with CuET or CBD+CuET combination as described in figure legend to measure the concentration of CuET in the cells. The media was then removed, cells were washed twice with 1x PBS, and 1x PBS was aspirated thoroughly. Cells were harvested by a scrapper and stored at -80°C. Cellular pellets were then homogenised with acetone and centrifuged 18 000 x g for 2 minutes at 4 °C, and the supernatant was transferred into a glass HPLC vial. The CuET complex was analysed by the HPLC-MS method described previously[8]. The calibration curve was used to calculate the quantity of the CuET complex.

3.11. Statistical analysis

Separated bar graphs of qPCR experiments are displayed as mean ± SD from 3 independent experiments. XY graphs of XTT assay, including error bars, are plotted as mean and error ± SD. All the figures depict 3 independent experiments, with each point presenting 5 replicates. 2D box plots of the quantitative microscopy analysis are plotted as median ± SD. All the experiments were carried out in 3 independent experiments. The figure depicting the 2D box plot represents a random selection from one of the experiments. The unpaired t-test was used to determine statistical significance, and the resulting p-value is displayed in graphs and figure legends. STATISTICA 13 or GraphPad Prism 8.0.1 were used for the graphical processing. GraphPad Prism 8.0.1 and 9.2.0 were used to calculate statistical significance and IC50 value.

3.12. Chemicals and antibodies

The following antibodies were used for immunoblotting: anti-β-actin (1:1000; Santa Cruz Biotechnology, cat. n.: sc-47778), anti-DDK (1:1000; OriGene, cat. n.: TA50011-100), anti-GAPDH (clone 1D4, 1:500; GeneTex, cat. n.: GTX78213), anti-histone H3 (1:2000; Cell Signaling, cat. n.: 4499P), anti-MTF1 (1:1000; NOVUS Biologicals, cat. n.: NBP1-

86380), anti-NPLOC4 (1:1000; NOVUS Biological, cat. n.: NBP1-82166), anti-Ubiquitin K48 (clone Apu 2, cat. n. 05-1307, Millipore) goat-anti mouse IgG-HRP (1:1000; GE Healthcare, NA931), goat-anti-rabbit (1:1000; GE Healthcare, NA934), donkey-anti goat IgG-HRP (Santa Cruz Biotechnology, sc-2020). The formulation of CuET (bis-diethyldithiocarbamate-copper complex) is directly synthesised in water in the presence of 1% bovine serum albumin, as described previously[8,308]. In short, 10 ml of 2,8mM CuET is prepared by adding 200µl of 280mM solution of sodium diethyldithiocarbamate trihydrate (DTC, Sigma Aldrich) and 28 µl of 1M $CuCl_2$ (Sigma Aldrich) into 1% solution of bovine serum albumin in ddH20 (Sigma Aldrich). The CuET formulation was stored for no more than a month at 4°C. The 10µM chelator bathocuproine disulfonic acid (Sigma Aldrich) was used for copper chelation and was added to cells just before the disulfiram (Sigma Aldrich) treatment. Finally, CBD [-(-)Cannabidiol] was ordered from Abcam (cat. n.: ab120448) and dissolved in methanol (Penta) to a concentration of 10 mM.

4. RESULTS

4.1. A high-throughput screen for modulators of CuET activity identified cannabidiol (CBD)

A high-throughput screening method was designed to identify biologically active molecules affecting the anticancer activity of the copper bis-diethyldithiocarbamate-copper complex (CuET). The screen was based on evaluating CuET's primary anticancer target, NPL4 protein. Mechanistically, cells treated with CuET undergo aggregation of NPL4 protein. Thus aggregated protein is immobilised in the nucleus and endoplasmic reticulum. The effect can be visualised using reporter cell lines ectopically expressing GFP-tagged NPL4 protein such as the U-2-OS-NPL4-GFP cells (established previously[8]). Immobilised NPL4-GFP protein was evaluated using high-throughput automated microscopy.

For the screening, the cells were pretreated for 17 hours with chemical compounds (proprietary library of 1282 biologically active compounds), each at 10μM concentration, and subsequently treated with 0.2μM CuET for 3 hours. After the treatment, the cells were pre-extracted using detergent (Triton X-100) to remove soluble cellular content. As a result, only solid/insoluble cellular fractions remained, including immobilised NPL4-GFP. The cells were then fixed and labelled with Hoechst 33342 to visualise nuclei in which aggregated NPL4 forms a typical insoluble pattern (Fig. 7). The nuclei signal was recognized by automated fluorescence microscopy and used as a mask to evaluate the corresponding NPL4-GFP signal (Fig. 8).

The screen identified several substances capable of attenuating CuET ability to aggregate NPL4 protein, including cannabidiol (CBD) among the strongest hits (Fig. 8).

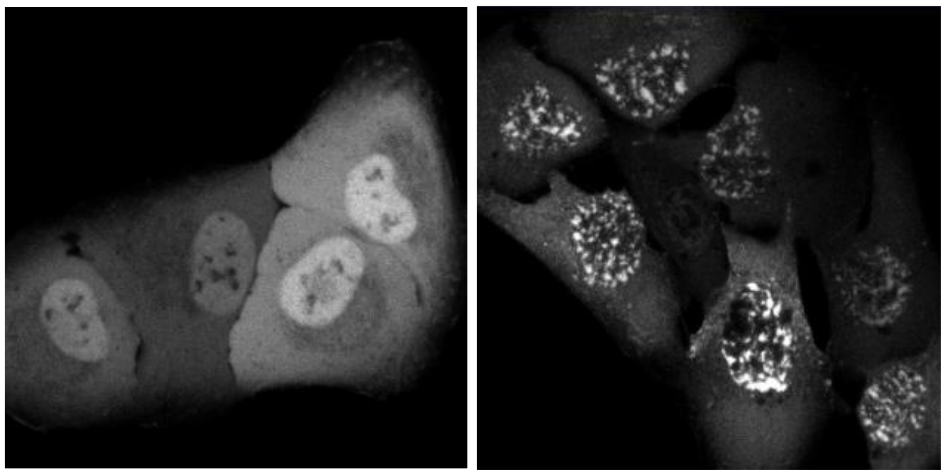

Fig. 7.: **NPL4-GFP signal in reporter U-2-OS cell line.** On the left mock-treated cells, the NPL4-GFP signal is homogeneously distributed within the nucleus and cytoplasm and can be washed away by the Triton X pre-extraction procedure. On the right, the cells are treated with 1μM CuET for 2 hours showing pre-extraction resistant NPL4 signal forming clusters within the nuclei and grainy pattern in the cytoplasms.

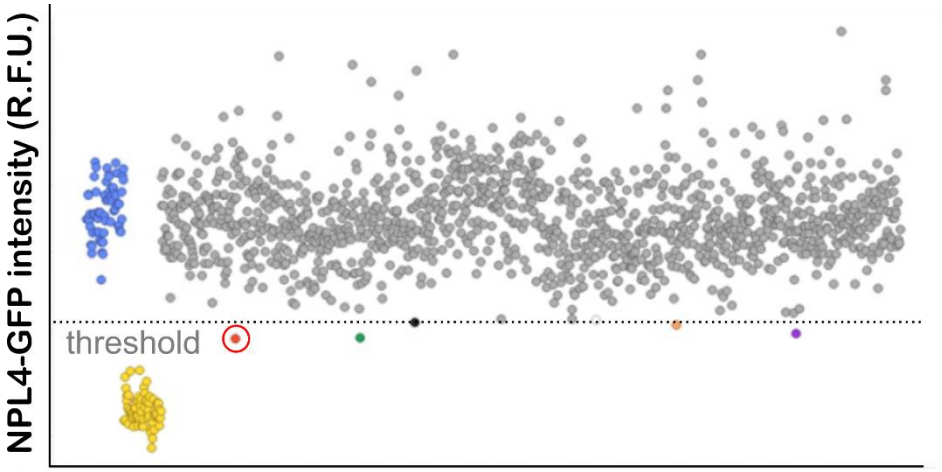

Fig. 8.: **Dot plot representing the results of high-throughput screening of the proprietary chemical library of biologically active compounds.** CuET-treated cells are depicted by blue, untreated cells are yellow, and cells treated with tested compounds passing the threshold of 0.5 % positive hits are coloured red, green, black, orange, and violet. The hit in the red circle represents cannabidiol (CBD) pretreatment. Cells were pretreated with compounds (10 μM) for 17 h and treated with 0.2μM CuET for 3 h.

Abbreviation: R.F.U. – Relative fluorescence units

The screen outcome was further verified by a more in-depth microscopic analysis of combined CBD+CuET treatment (Fig. 9A, 9B). The next aim was to confirm if the CBD-mediated protective effects also rescue endogenous NPL4. The rescue effect of endogenous NPL4 was confirmed in U-2-OS and MDA-MB-231 cell lines using the immunoblotting technique (Fig. 9C, 9D). The most crucial question was whether the observed effects also project to the modulation of CuET's cellular cytotoxicity. Indeed, the cells pretreated with CBD survive better than cells treated with CuET alone. For the toxic evaluation, the XTT assay was performed in three cell lines: U-2-OS, MDA-MB-231, and hTERT RPE-1 (Fig. 9E, Fig. 16B, and 16C as part of the following siRNA combined experiments).

The proteotoxic stress markers accompanying the CuET-promoted toxicity[8] were also analysed. For example, combined CBD+CuET treatment led to the decreased accumulation of K48 polyubiquitinated (Ub K48) proteins. This marker of impaired proteasomal degradation was detected by immunoblotting in U-2-OS and MDA-MB-231 cell lines (Fig. 10A, 10B). Additionally, it was observed that the mRNA expression of the HSP70 (*HSPA1A*), which CuET strongly induces, had also decreased after CBD pre-treatment, as depicted in U-2-OS (Fig. 10C).

These results confirm that CBD is a potent antagonist of CuET-mediated cellular responses, including toxicity.

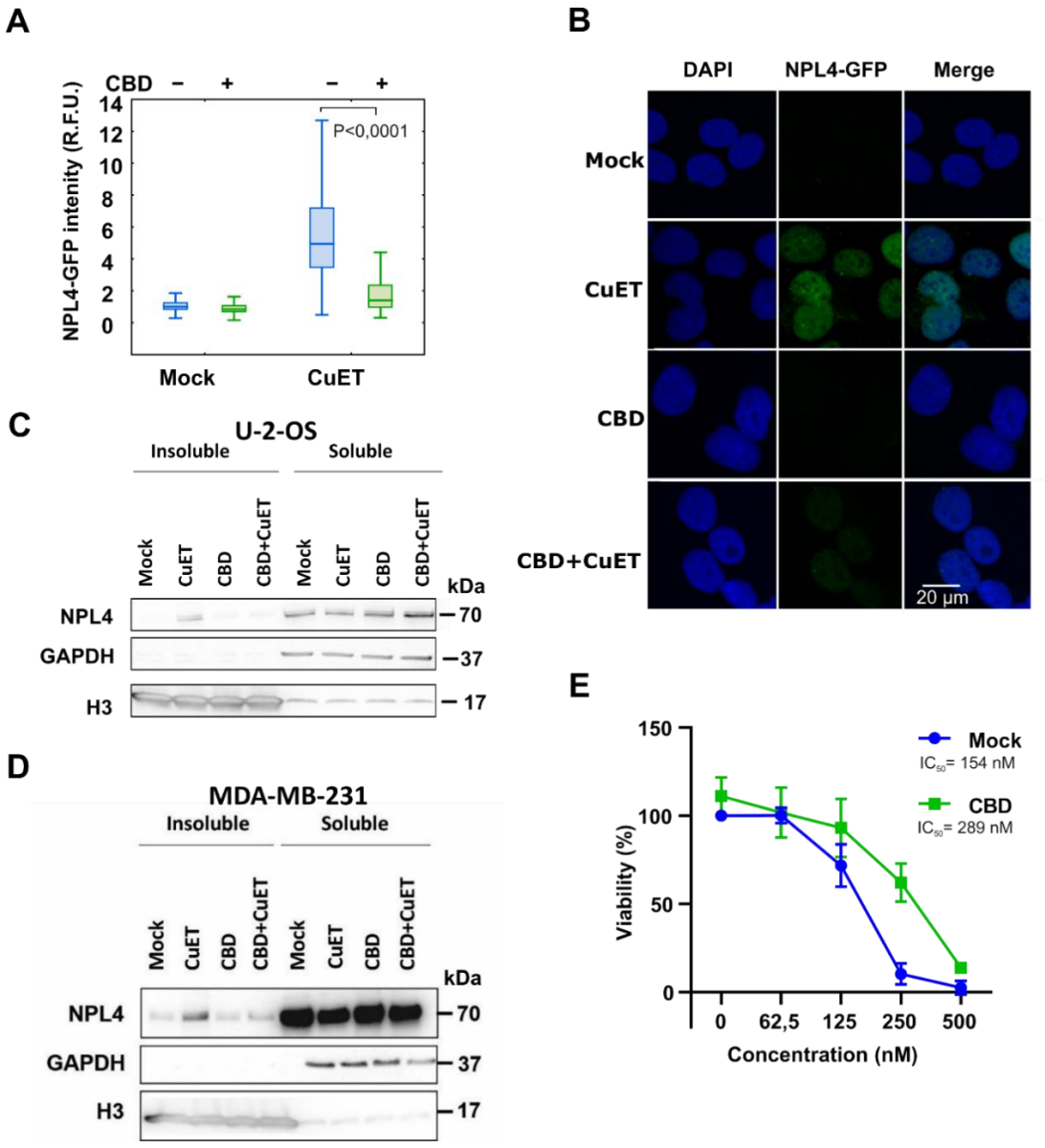

Fig. 9.: **Cannabidiol (CBD) protects cells from bis-diethyldithiocarbamate-copper complex (CuET).** (A, B) A microscopy-based analysis confirmed the most robust hit from the screen, CBD. Cells pretreated with combined CBD+CuET treatment accumulate less NPL4-GFP than cells treated with CuET alone. U-2-OS-NPL4-GFP cells were pretreated with 10µM CBD for 17 h, and treated with 0.2µM CuET for 3 h. For P value calculation, a two-tailed t-test was used. The figures show results from one of three independent experiments. The size of the scale bar is 20 µm. (C, D) Cells after the combined treatment accumulate less endogenous NPL4 than cells treated with CuET alone. U-2-OS and MDA-MB-231 cells were pretreated with 10µM CBD for 17 h and treated with 0.2µM CuET for 3 h. Cells were fractionated and analysed using a western blot. The figure shows one of three independent experiments. (E) U-2-OS cells treated with combined CBD+CuET treatment are more resistant than cells treated with CuET only, as observed by the XTT assay. Cells were pretreated with 10µM CBD for 17 h and treated with 10µM CBD and increasing concentration of CuET for 72 h. The results represent three independent experiments' mean and standard deviation.

Abbreviation: IC50 – half-maximal inhibitory concentration, R.F.U. – Relative fluorescence units

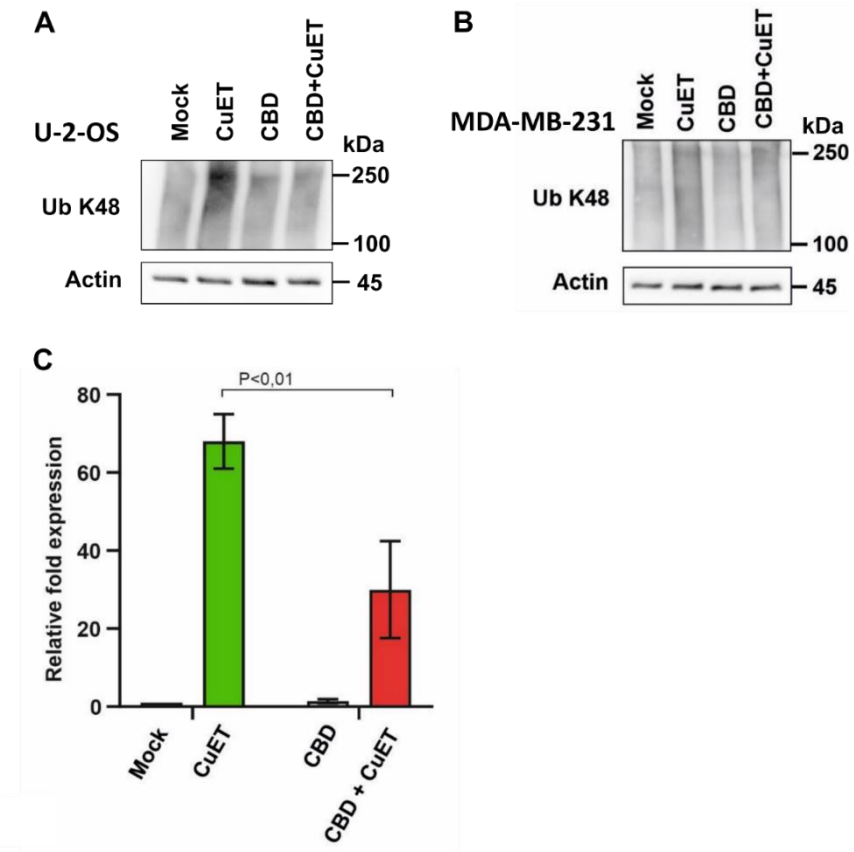

Fig. 10.: **Cannabidiol (CBD) attenuates proteotoxic stress response induced by bis-diethyldithiocarbamate-copper complex (CuET).** (A, B) Western blot images show lower induction of polyubiquitinated proteins in cells with combined CBD+CuET treatment compared to CuET-only-treated cells. U-2-OS and MDA-MB-231 were pretreated with 10μM CBD and then treated for 3h by 0.2μM CuET. The figure shows one of three independent experiments. (C) mRNA expression of HSP70 (*HSPA1A*) is attenuated after CBD pretreatment. U-2-OS cells were pretreated with 10μM CBD for 17 h, followed by 0.2μM CuET for 3 hours, and evaluated by quantitative polymerase chain reaction. T-test was used for P-value calculations. The result represents three independent experiments' mean and standard deviations.

4.2. CBD and also CuET induce metallothionein expression

The next question addressed was the mechanism behind the CBD-promoted protection of cells from CuET. The most straightforward explanations offered direct molecular interaction leading to chemical alteration/inactivation of CuET molecule or altered cellular uptake/efflux. Both possibilities were addressable by mass-spectrometry of CuET's level. However, there was no significant impact on CuET levels in the presence of CBD in the cultivation medium, excluding the direct chemical interaction (Fig. 11A). Neither uptaker/efflux explanation worked, as CuET's cellular content among Mock- or CBD-pre-treated samples did not show any significant differences (Fig. 11B).

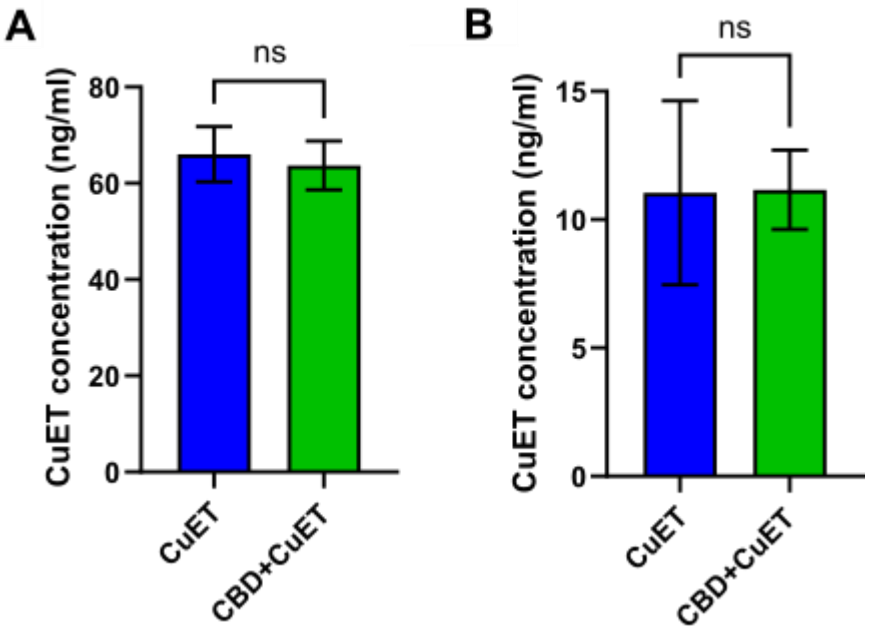

Fig. 11.: **Cannabidiol (CBD) does not alter bis-diethyldithiocarbamate-copper complex (CuET) chemically, nor affects its cellular uptake.** A) CBD does not influence the amount of CuET in media, as analysed by high-pressure liquid chromatography combined with mass spectrometry HPLC-MS. 10μM CBD and 0.2μM CuET were added to the media and incubated in parallel with cells. B) Intracellular CuET amount was unchanged with or without CBD pretreatment. U-2-OS cells were pretreated with 10μM CBD for 17 hours, followed by 0.2μM CuET treatment for 3 hours. Differences in CuET level are not significant, as calculated by T-test. The results represent three independent experiments' mean and standard deviation.

Abbreviation: n.s. – not significant

The other explanatory mechanisms could be found in publications describing CBD's biological effects and molecular targets. Among such CBD's promoted responses is the activation of the so-called metallothionein (MT) pathway[193,309]. Indeed, quantitative PCR confirmed CBD-induced overexpression of MTs in U-2-OS and MDA-MB-231. Interestingly, both tested MTs, MT-1A and MT-2A, also responded to CuET treatment by strong upregulation. In combined CBD+CuET treatment, MTs expression was even higher (Fig. 12). These initial results suggested that MTs might be responsible for the CBD-promoted protective effects against CuET.

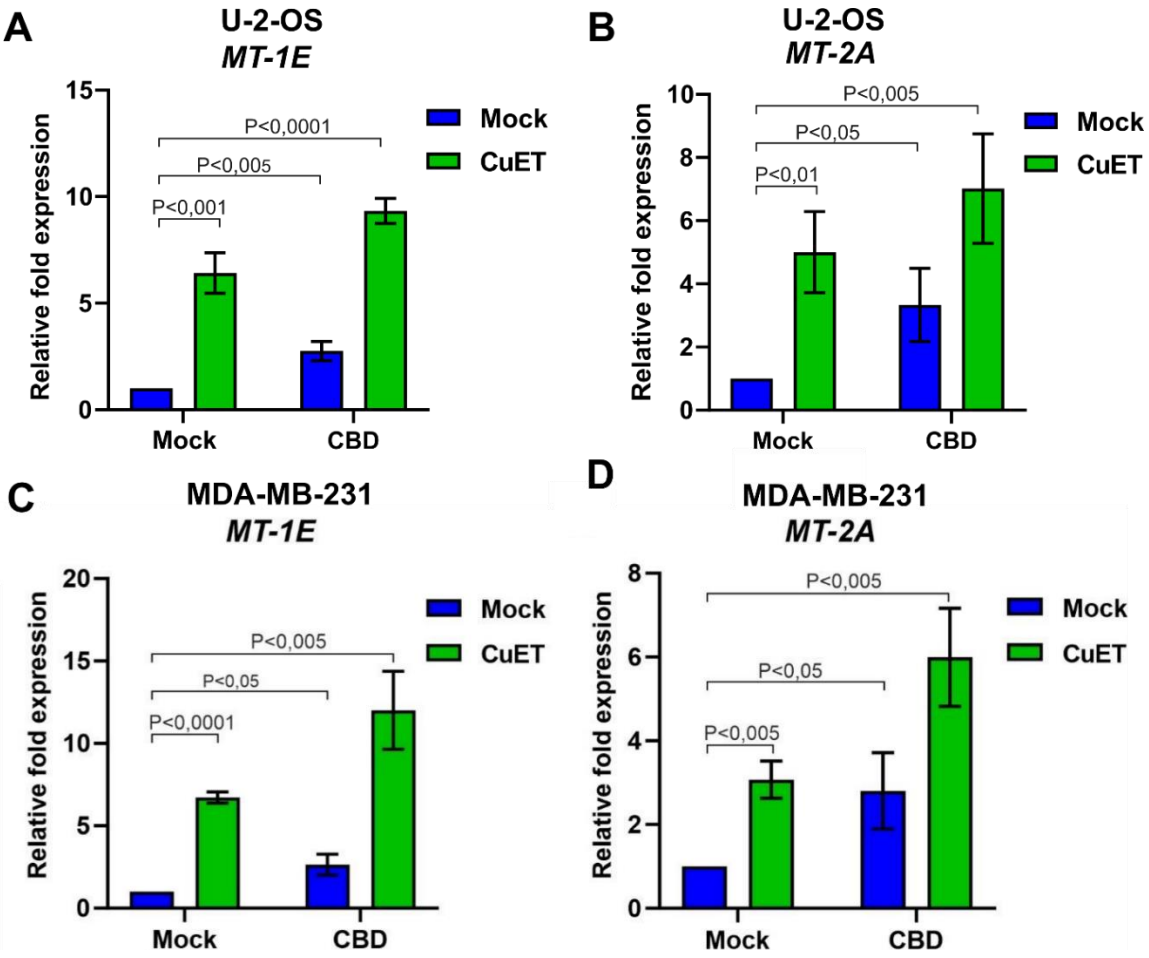

Fig 12.: **Cannabidiol (CBD) and bis-diethyldithiocarbamate-copper complex (CuET) induce MT-1E and MT-2A mRNA expression.** (A, B) CBD and CuET induce the expression of *MT-1E* and *MT-2A* mRNA in U-2-OS cells. (C, D) CBD and CuET induce the expression of *MT-1E* and *MT-2A* mRNA in MDA-MB-231 cells. Both cell lines were pretreated with 10μM CBD for 17 h and treated with 0.2μM CuET for 3 h. For all four charts, a two-tailed t-test was used for P-value calculation. The result represents the mean and standard deviation of three independent experiments.

4.3. Metallothioneins protect cells from CuET-mediated toxicity

Previous experiments demonstrated that CBD reduces the cytotoxic effects of CuET. In addition, it was confirmed that MTs, which are in charge of maintaining zinc ion homeostasis and detoxifying cells by binding heavy metals, are induced by CBD. Importantly, CuET also induced MTs overexpression, suggesting MTs' role in the CBD-mediated rescue effect. On that account, a set of experiments that directly altered the MT pathway was performed. The pathway was manipulated in both directions using complementary siRNA-based and ectopic expression strategies.

MTF1 is the central transcriptional regulator of MTs expression; therefore, it was selected for the knockdown. Interestingly, MTF1-silenced cells acquired high sensitivity to CuET compared to control-silenced cells in three different cellular models (Fig. 13A, 13B, 13H-K 16C, 16E as part of the following siRNA combined experiments). The result was further underlined by increased accumulation of NPL4-GFP protein in the U-2-OS-NPL4-GFP model, also by increased accumulation of Ub K48 proteins in U-2-OS and MDA-MB-231 cancer cell lines, and finally by increased expression of HSPA1A in U-2-OS cells (Fig. 13C-G).

For the overexpression experiments, MT-2A (DDK/flag-tagged) was chosen. As expected, the overexpression was accompanied by increased CuET resistance compared to cells transfected with empty vectors (Fig. 14A, 14F). Similarly, as with MTF1 knockdown, the result was strengthened by further experiments showing lower immobilisation of NPL4-GFP protein in U-2-OS-NPL4-GFP cell line, decreased accumulation of Ub K48 proteins, and decreased expression of HSPA1A in U-2-OS cells (Fig. 14B-E).

Additionally, any potential direct interactions between CuET and MTs were closely examined in the U-2-OS cell line, stably overexpressing turboGFP-tagged MT-2A. Following CuET treatment, the cells were pre-extracted, DAPI-stained, and then microscopically visualised. Strikingly, CuET turned MT-2A-GFP protein into a pre-extraction resistant form resembling the effect CuET has on the NPL4 protein (Fig. 15). This experiment strongly suggests a direct interaction between CuET and MTs.

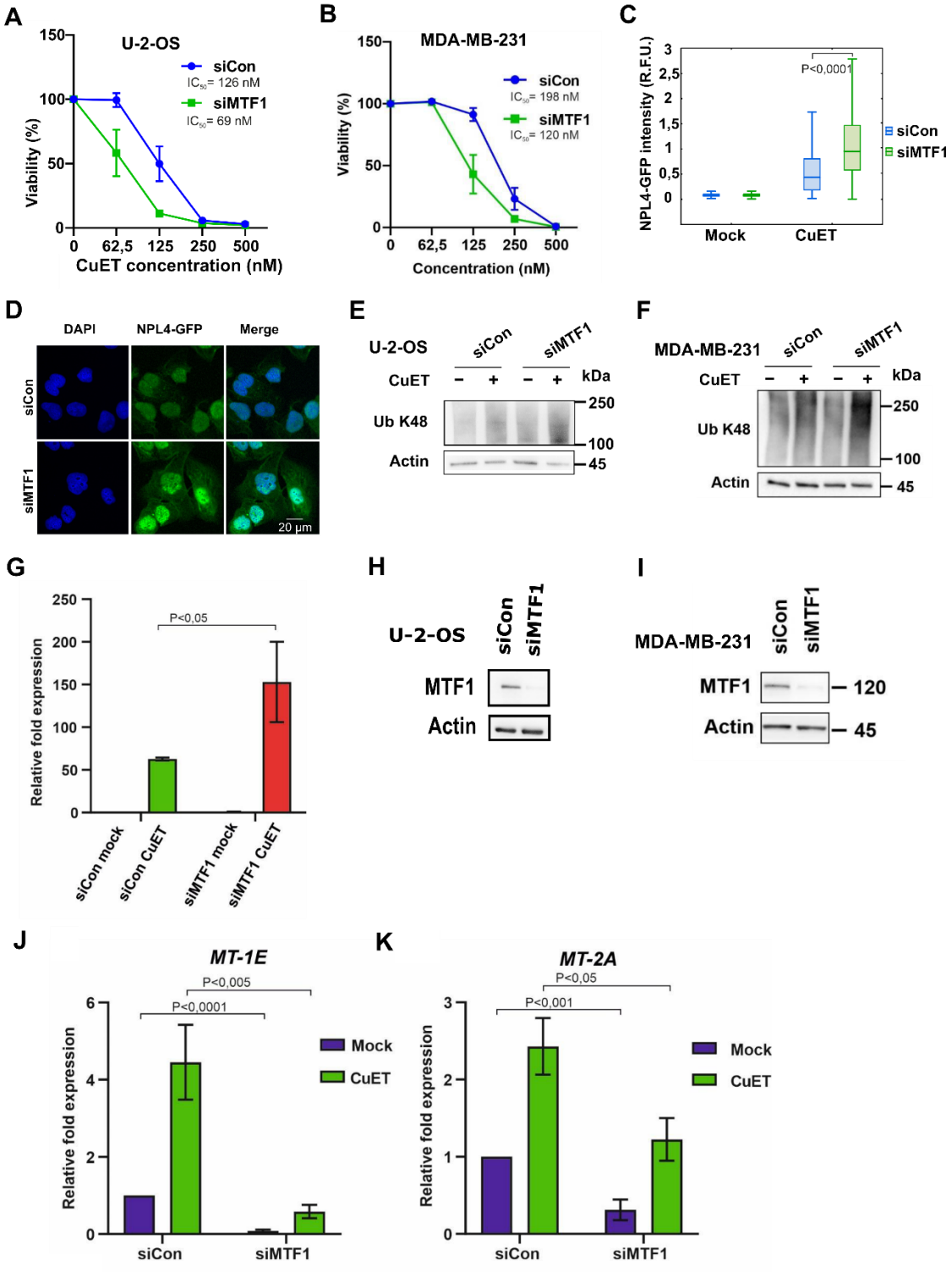

Fig. 13.: **MTF1 silencing modulates the cellular responses to bis-diethyldithiocarbamate-copper complex (CuET) in U-2-OS cells.** Description on the next page.

Fig. 13.: **MTF1 silencing modulates the cellular responses to bis-diethyldithiocarbamate-copper complex (CuET) in U-2-OS cells.** (A, B) MTF1 knockdown sensitises cells to CuET, as analysed by XTT assay. U-2-OS and MDA-MB-231 cells were treated with increasing concentrations of CuET for 72 h. The result represents the mean and standard deviation of three independent experiments. (C, D) MTF1-silenced cells accumulate more NPL4-GFP after CuET than control-silenced cells. U-2-OS-NPL4-GFP cells were treated with 0.2μM CuET for 3 h, followed by microscopy-based analysis. The scale bar has a size of 20 μm. For P value calculation, a two-tailed t-test was used. The results represent one of three independent experiments. (E, F) MTF1-silencing increases the accumulation of K48 polyubiquitinated proteins after CuET treatment. U-2-OS and MDA-MB-231 cells were treated with 0.2μM CuET for 3 h and analysed using immunoblotting. The figure shows one of three independent experiments. (G) MTF1-silencing increases mRNA expression of HSP70 (*HSPA1A*). Cells were treated with 0.2μM CuET for 3 hours and analysed by quantitative polymerase chain reaction. T-test was used for P-value calculations. The result represents three independent experiments' mean and standard deviation. (H, I) WB-based verification of knockdown efficacy of the MTF1 transcription factor in the U-2-OS and MDA-MB-231 cell lines. The result represents one of three experiments. (J, K) Knockdown of MTF1 transcription factor decreases *MT-1E* and *MT-2A* mRNA levels in the mock-treated and CuET-treated cells. Transfected U-2-OS cells were treated with 0.2μM CuET for 3 hours and evaluated by qPCR. T-test was used for P-value calculations. The result represents the mean and standard deviation of three independent experiments.

Abbreviation: IC50 – half-maximal inhibitory concentration, R.F.U. – Relative fluorescence units

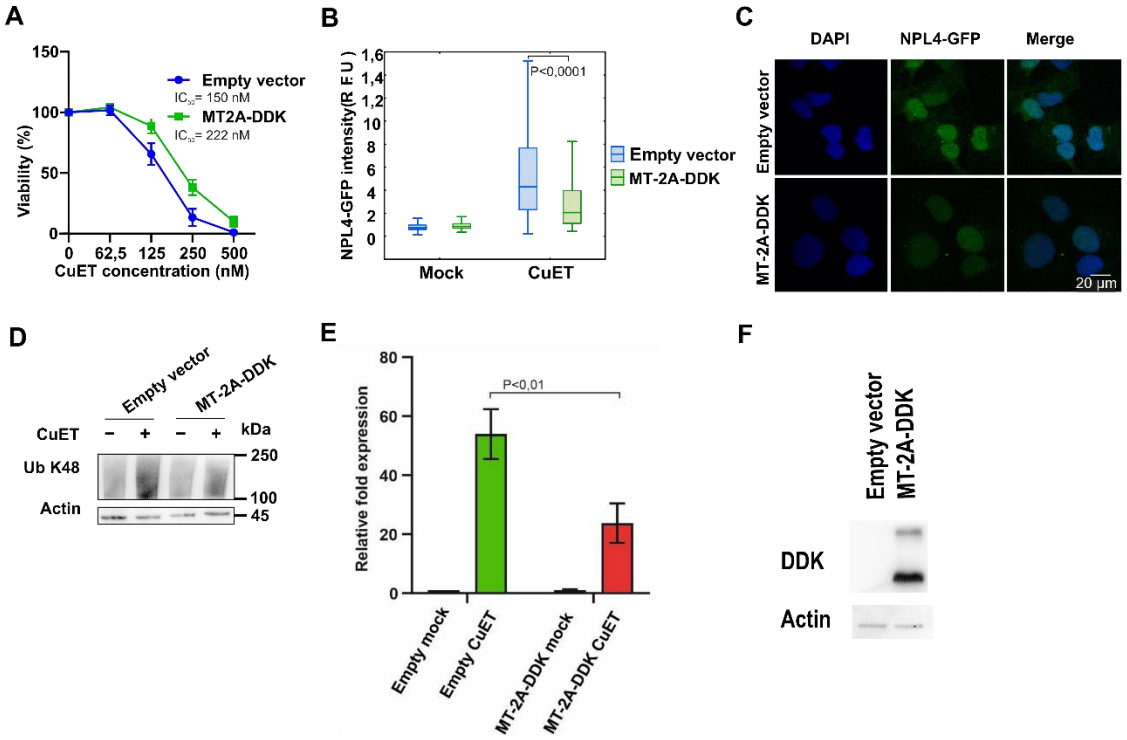

Fig. 14.: **Ectopic overexpression of MT-2A protects U-2-OS cells from bis-diethyldithiocarbamate-copper complex (CuET).** (A) MT-2A-DDK-overexpression makes cells more resistant to CuET compared to empty vector-transfected cells. Cells were treated with increasing concentrations of CuET for 72 h. Viability was evaluated by XTT assay. The result represents three independent experiments' mean and standard deviation. (B, C) MT-2A-DDK-overexpressing cells immobilise less NPL4-GFP, as shown in the chart and corresponding microscopic image (20 µm scale bar). Accumulate significantly less of the non-dissolvable NPL4 as depicted by microscopic images (20 µm scale bar). Cells were treated with 0.2µM CuET for 3 h and microscopically analysed. A two-tailed t-test was used for P-value calculation. The results represent one of three independent experiments. (D) MT-2A-DDK-overexpressing cells accumulate a lower amount of K48 polyubiquitinated proteins after CuET. Cells were treated with 0.2µM CuET for 3 h and analysed by immunoblotting. The figure shows one of three independent experiments. (E) mRNA expression of HSP70 (HSPA1A) is decreased in MT-2A-DDK-overexpressing cells. Cells were treated with 0.2µM CuET for 3 hours and analysed by quantitative polymerase chain reaction. T-test was used for P-value calculations. The result represents three independent experiments' mean and standard deviation. (F) WB-based confirmation of MT-2A-DDK level in U-2-OS cells after transfection of plasmid containing Myc-DDK-tagged MT-2A. The result represents one of three experiments.

Abbreviation: IC50 – half-maximal inhibitory concentration, R.F.U. – Relative fluorescence units

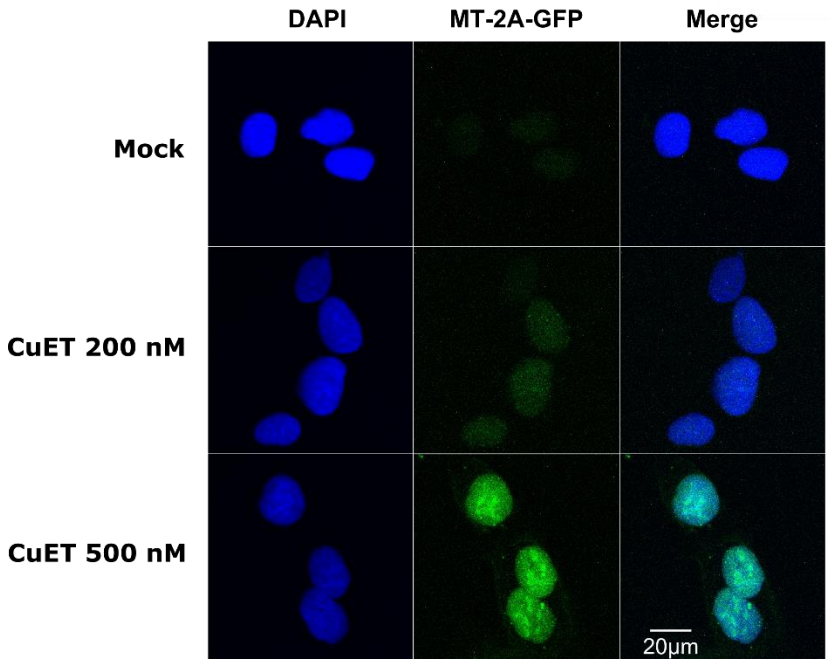

Fig. 15.: **Bis-diethyldithiocarbamate-copper complex (CuET) treatment immobilizes MT-2A-GFP in U-2-OS cells.** Microscopic images of MT-2A-GFP protein showing immobilization after CuET treatment. Cells were treated with 0.2 and 0.5μM CuET for 3 h and pre-extracted with Triton X-100 (20 μm scale bar). The figure represents one of two independent experiments.

To further confirm that the MT pathway is in charge of the CBD-mediated rescue effect, MTF1-silenced cells were employed. As expected, CBD failed to rescue MTF1-silenced and CuET-treated cells (Fig. 16A). This experiment directly linked CBD, MTs, and CuET. Therefore, three distinct cell lines were tested, including the primary hTERT RPE-1 cell line, with identical results (Fig. 16A-C, 16E). CBD was further tested also in MT-2A-silenced cells. This experiment was performed to clarify that particular MT could stand behind the effect and not MTF1 itself. Compared to control-silenced cells, the CBD-mediated rescue effect was substantially diminished in MT-2A-silenced cells (Fig. 16D, 16F). In this case, however, CBD still had a partial rescue effect, which could be explainable by the presence of the remaining non-silenced MTs.

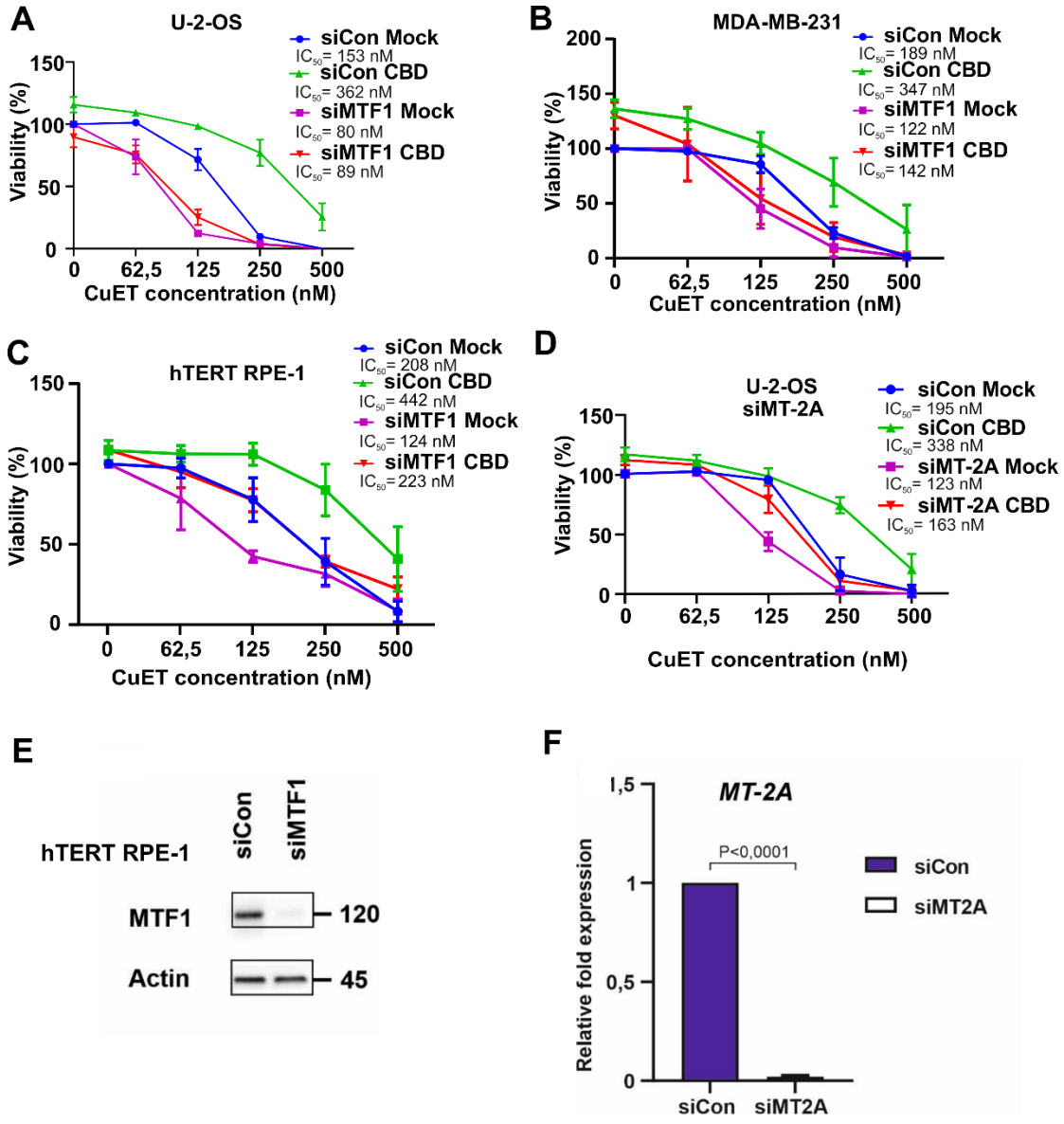

Fig. 16.: **Cannabidiol (CBD)-mediated protection against bis diethyldithiocarbamate-copper complex (CuET) depends on metallothioneins.** (A, B, C) MTF1-silenced cells are not protected by CBD pretreatment from CuET toxicity. Cell lines were pretreated with 10μM CBD for 17 h, treated with 10μM CBD, and increasing concentration of CuET for 72 h (hTERT RPE-1 were treated for 24 h), and evaluated by XTT assay. The result represents three independent experiments' mean and standard deviation. (D) MT-2A-silencing partially hinders CBD-mediated protection from CuET toxicity. Cells were pretreated with 10 μM CBD for 17 h, treated with 10 μM CBD and an increasing concentration of CuET for 72 h, and evaluated by XTT assay. The result represents three independent experiments' mean and standard deviation. (E) WB-based verification of knockdown efficacy of the MTF1 transcription factor in hTERT RPE-1 cell line. The result represents one of three experiments. (F) siRNA-based silencing of metallothionein MT-2A verified by qPCR in U-2-OS cells. A T-test was used for P-value calculation. The result represents the mean and standard deviation of three independent experiments.

Abbreviation: IC50 – half-maximal inhibitory concentration

4.4. CBD/MTs-promoted rescue effect involves disulfiram in case of the presence of Cu^{2+} ions in the culture medium

The project was enclosed with experiments linking CBD, MTs, and DSF. There is persisting debate in the scientific literature about the mechanism behind the anticancer effects of DSF. Besides NPL4 targeted via CuET metabolite, multiple cancer-relevant direct or undirect DSF targets were suggested[310,311]. However, other DSF anticancer targets hypothesis seems improbable because MTF1-silenced cells show increased sensitivity to DSF similar to CuET (Fig. 17). Moreover, this sensitivity is entirely dependent on the presence of Cu^{2+} ions in the culture medium as shown by experiment with BCDS copper chelator pretreatment which suppressed any toxic effect of DSF regardless of MTF1's status (Fig. 17). These data add another prove that DSF's toxicity towards cancer cells is dependent on the presence of copper ions and thus the formation of CuET in the medium[312]. Therefore, CBD-MTs mediated protection of cancer cells might be highly relevant also for DSF usage in clinical oncology.

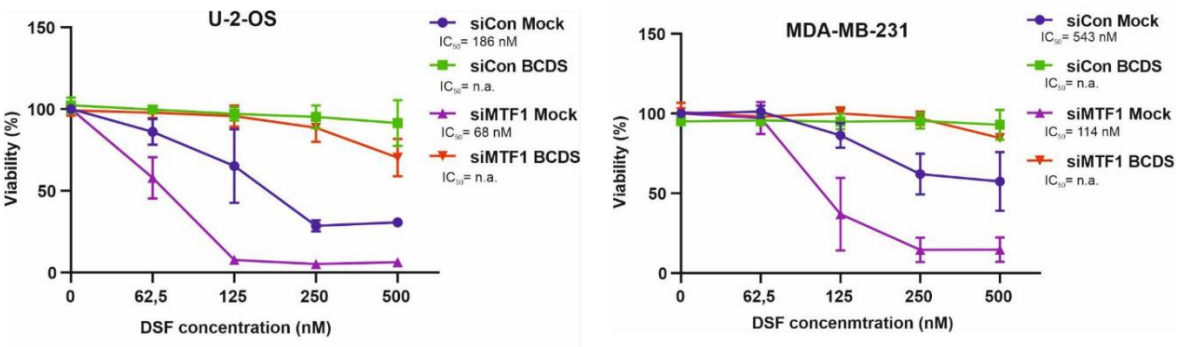

Fig. 17.: **Metallothioneins target bis-diethyldithiocarbamate-copper complex (CuET), not disulfiram (DSF).** Chelation of copper prevents the formation of CuET molecules and leads to resistance of cells in the presence of DSF. MTF1-silence cells also become resistant. Cell lines U-2-OS and MDA-MB-231 were treated with an increased concentration of DSF for 72 hours and evaluated by XTT assay. 10μM copper chelator bathocuproine disulfonic acid (BCDS) was added shortly before the DSF treatment. The result represents three independent experiments' mean and standard deviation.

5. DISCUSSION

Drug resistance is a growing issue in oncology, forcing the development of new drugs or the repurposing of old ones. It was previously discovered that together with principal cellular adaptations and selection, drug resistance could also be affected by patients' nutrition and/or other drug supplementation during the therapy[9]. Disulfiram (DSF), a drug initially developed to treat alcohol abuse, has recently become a hot candidate for repurposing in cancer therapy. DSF was demonstrated to induce proteotoxic stress via its metabolite, bis-diethyldithiocarbamate-copper complex (CuET), targeting NPL4 protein with an irreplaceable role within the ubiquitin-proteasome system (UPS)[8]. CuET in nanomolar concentration induces massive proteotoxic stress by NPL4 aggregation and weakening the UPS. Despite the potent ability of DSF/CuET to effectively kill cancer cells, results in clinical trials were rather ambiguous[313]. This work was dedicated to finding a possible explanation for some DSF-treated cancer patients' insufficient response.

A high-throughput screening strategy was applied to find compounds interfering with CuET biological activity for that purpose. The screening method was based on evaluating the primary CuET target, the NPL4-GFP protein, by automated microscopy. Among the most potent hits was identified cannabidiol (CBD), a component of the marijuana plant.

CBD is a highly relevant substance in cancer due to its relatively common usage among oncological patients[10,11]. One-fifth of the patients prefer pure CBD or CBD-dominant products[11]. The scale is so massive that one-third of oncology physicians acknowledge discussing marijuana usage as an adjuvant with their patients[314]. Moreover, marijuana and cannabinoids have been extensively studied over the past few decades, revealing numerous clinically relevant cellular targets[199,246,315]. Notably, CBD, under the commercial name Epidiolex, received FDA approval for treating seizures caused by two kinds of epilepsy and tuberous sclerosis complex[171,172]. Apart from the prescription form, CBD is widely available over-the-counter. Due to its generally safe pharmacological profile, CBD is widely available in many countries worldwide in foods, beverages, tobacco-like products, and cosmetics[12]. It is important to note that CBD or marijuana in various forms is generally accepted as

a treatment for attenuation of chemotherapy-induced adverse effects, including pain, nausea, vomiting, loss of appetite, and depression. Some studies suggest it may even work as a cancer therapy potentiator[62,199,237,245,246]. For example, Nabilone and Dronabinol (both synthetic cannabinoids) are already FDA-approved for chemotherapy-induced nausea and vomiting[174]. Sativex is one more cannabinoid-based drug containing CBD together with Δ^9-THC, which is for a decade under the evaluation against cancer-related pain[316]. Pure CBD is planned to be extensively tested for its potential ability to reduce chemotherapy-induced side effects[279–281,317]. With such solid clinical relevance, finding the mechanism behind the CBD-promoted protective effect against CuET was chosen for a detailed experimental examination.

Experimental data collected within this work link the CBD-promoted protective effects to the metallothionein (MT) pathway. MTs are small proteins acting in a cell as a chelating agent, helping to balance zinc homeostasis and detoxifying cells by heavy metals removal[291,292]. Additionally, it has been previously reported that MTs are responsible for chemotherapy resistance of some drugs and are suggested as potential markers for chemotherapy efficacy[291,292,297,298].

Interestingly, an independent group recently reported protective effects of the MT pathway against DSF[309]. The group screened for genetic determinants of sensitivity towards various anticancer drugs and revealed that cells with disrupted MT pathways are susceptible to DSF treatment. The authors proposed possible interaction between MTs and DSF. However, experiments performed in this work clearly show that DSF's cellular toxicity entirely depends on the copper ions and formation of the CuET molecule, which is, in fact, the MTs' target. Moreover, CuET contains divalent cupric cation, which explains the interference through suggested chelation by MTs. It is essential to underline that the presence of copper ions is an important factor for successful DSF-mediated anticancer therapy. Some of the clinical trials even combine DSF with copper supplementation. Also, in some cases, the copper pool can be decreased in patients with celiac disease and in patients with zinc supplementation, which was shown to affect copper uptake negatively[318,319].

Overall, the data presented in this work uncovers an essential mechanism of cellular resistance against CuET molecule (Fig. 18) and provides important clinically relevant

messages. First of all, it is the usage of cannabis-derived products, including CBD, which should be avoided during the anticancer treatment based on DSF. One can even raise the question of whether the unwanted CBD-promoted protective effect is not affecting more types of anticancer therapies, such as platinum-based. Last but not least is the applicability of the MT pathway status as a predictive marker for successful DSF-based treatment. Moreover, the MT pathway is chemically targetable, offering potential options for combined therapies. For example, compound APTO-253 was under clinical testing as a c-Myc inhibitor, which also inhibits MTF1[320].

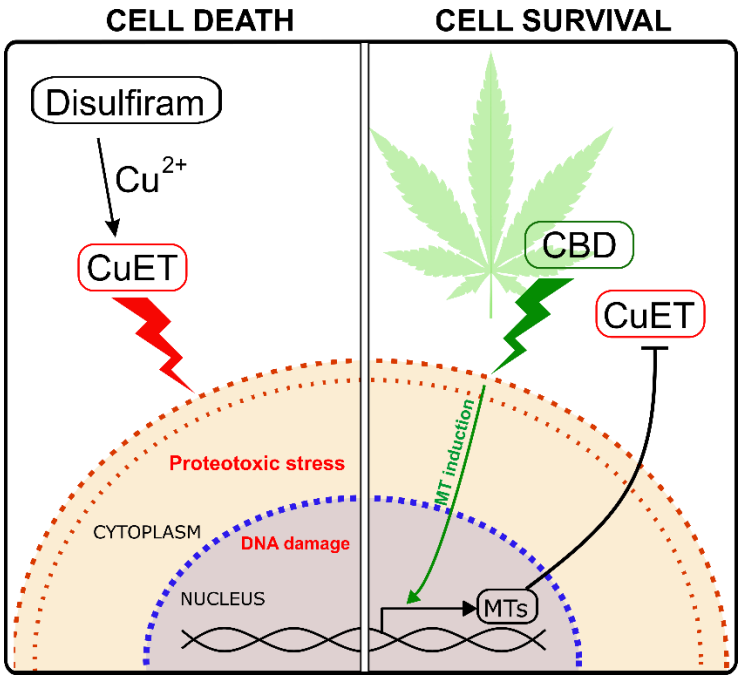

Fig. 18: A complex of copper and diethyldithiocarbamate, CuET, a product of disulfiram (DSF) metabolisation, targets cancer cells via proteotoxic stress and DNA damage. Concomitant usage of cannabis-derived products attenuates the effect of CuET by promoting the induction of metallothioneins, which bind to CuET and interfere with its cellular activities.

6. CONCLUSION

In conclusion, the patients receiving disulfiram (DSF) therapy should avoid using cannabidiol- (CBD)-containing products concurrently due to identified interference of CBD and bis-diethyldithiocarbamate-copper complex (CuET) which is the anticancer metabolite of DSF. This discovery might also explain why the clinical trials aimed at repurposing DSF in oncology were beneficial only for some patients. Metallothioneins (MTs), which expression is triggered after CBD treatment, not only protect the cancer cells from CuET, but their status might also serve as predictive biomarkers for DSF therapy. Patients with lower expression of MTs in cancer tissues should be more suitable for successful therapy. Presented data also suggest that combined treatments, including DSF and drugs targeting the MT pathway, might become a potent anticancer strategy.

7. ABBREVIATIONS

5-HT1A/2A/3A	5-hydroxytryptamine receptor 1A/2A/3A
ABCB11/G2	ATP binding cassette subfamily B member 11/G member 2
ATF4/6	Activating transcription factor 4/6
Ca^{2+}	Calcium
CB1/2	Cannabinoid receptor 1/2
CBD	Cannabidiol
CBN	Cannabinol
CNS	Central nervous system
CuET	bis-diethyldithiocarbamate-copper complex
Cu^{2+}	Copper
CYP450	Cytochrome P450
DSF	Disulfiram
DUB	Deubiquitinate
eIF2α	Eukaryotic initiation factor 2 subunit-α
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FAAH	Fatty acid amide hydrolase
FDA	Food and Drug Administration
GABA-A	Gamma-aminobutyric acid type A
GlyR	Glycine receptor
GPR18/55	G protein-coupled receptor 18/55
HSP	Heat shock protein
IC50	Half-maximal inhibitory concentration
IRE1α	type 1 ER transmembrane protein kinase/endoribonuclease
K48	Lysin 48
MM	Multiple myeloma
MT	Metallothionein
MTF1	Metal-responsive element-binding transcription factor 1
NPL4	NPL4 - Nuclear protein localisation protein 4

n.s.	Not significant
PPARγ	Peroxisome proliferator-activated receptor gamma
PERK	PERK (Protein Kinase RNA-Like ER Kinase
p-gp	P-glycoprotein
RPN	Regulatory particle non-ATPase
RPT	Regulatory particle AAA+ ATPase
R.F.U.	Relative fluorescence units
THC	Tetrahydrocannabinol
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPM8	Transient receptor potential melastatin 8
TRPV1/2/3/4	Transient receptor potential vanilloid 1/2/3/4
VCP/p97	Valosin-containing protein
Ub	Ubiquitin
UFD1	Ubiquitin recognition factor in ER-associated degradation protein 1
UGT	UDP-Glucuronosyltransferases
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
PQC	Protein quality control
PS	Proteotoxic stress
XBP1	X-box-binding protein 1
Zn^{+}	Zinc
α2-AR	Alpha-2 adrenergic receptor
δ-/κ-/μ-OPR	Delta-/kappa-/mu-opioid receptor

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9.2. CONFERENCE LECTURES AND POSTER PRESENTATIONS

ORAL TALKS:

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