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**Vliv diethyldithiokarbamátu mědi (CuET) na aktivaci lymfocytů a protinádorovou odpověď**

# BAKALÁŘSKÁ PRÁCE

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# PODĚKOVÁNÍ

# Bibliografická identifikace

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Abstrakt

Tato bakalářská práce je zaměřena především na studium imunitní odpovědi proti nádorovým onemocnění, která vzniká v důsledku reakce na antigen exprimovaný nádorovými buňkami. Je zkoumán vliv metabolitu disulfiramu na aktivitu imunitních buněk a na možné změny v prezentaci antigenu samotnými nádorovými buňkami. Teoretická část stručně pojednává o imunitním systému, o rakovině jako nemoci a o tom, jak se imunitní systém podílí na destrukci nádorových buněk. Teoretickou část uzavírá kapitola o disulfiramu a komplexu CuET, který vzniká jeho metabolizací, a informace o protirakovinných účincích těchto látek. V experimentální části se snažíme určit hlavní faktor zodpovědný za změny cytotoxicity, zda se jedná o stimulaci imunitních buněk, možnou změnu prezentace antigenu nádorovým buňkám nebo obojí současně. Experimentální část se dále zabývá fosforylací signálů po ošetření CuET. Bylo zjištěno, že předběžné ošetření lymfocytů CuET významně zvyšuje cytotoxickou odpověď proti buněčným liniím myšího melanomu B16F10. Po ošetření CuET došlo také k významnému zvýšení množství fosforylovaných tyrozinových proteinů. To naznačuje, že ošetření CuET vede k regulaci signálních kaskád.

Klíčová slova      CuET, disulfiram, tumor antigens, cytotoxic T cells, antitumor immunity, pTyr-signalization

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Abstract

This bachelor thesis is primarily focused on the role of the immune cells in cancer, in response to antigen expressed by tumor cells. It examines the effect of the metabolite disulfiram on the activity of immune cells and on possible changes in the antigen presentation of cancer cells themselves. The theoretical part briefly discusses the immune system, cancer as a disease and how the immune system is involved in the destruction of cancer cells. The theoretical part concludes with a discussion of disulfiram and the CuET complex that is formed by its metabolism, as well as the anti-cancer effects of these substances. In the experimental part we try to identify the main factor responsible for the changes in cytotoxicity, whether it is stimulation of immune cells, possible alteration of antigen presentation to tumor cells or both at the same time. The experimental part further addresses signal phosphorylation after CuET treatment. Pre-treatment of lymphocytes with CuET was found to significantly enhance the cytotoxic response against B16F10 mouse melanoma cell lines. There was also a significant increase in the amount of phosphorylated tyrosine proteins after CuET treatment. This indicates, that CuET treatment results in the regulation of signaling cascades.

| | |
|---|---|
| Keywords | CuET, disulfiram, nádorové antigeny, cytotoxické T buňky, protinádorová imunita, pTyr-signalizace |
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# TABLE OF CONTENS

# AIMS OF THE THESIS

- Gather literary resources and elaborarate the theoretical review on the topic of the bachelor thesis.

- Stimulation of the tumor cell line with CuET of different contrentations and time points to induce the expression of proteins that serve as antigens for immune cell recognition. Antigen recognition will lead to a higher cytotoxic response.

- Another objective is to determine whether CuET is able to stimulate lymphocyte cell signaling, which increases the cytotoxic response.

# 1 INTRODUCTION

Cancer is a complex disease that is the second leading cause of death worldwide. In recent years, analysis of epidemiological data on various drugs has revealed that disulfiram, has some anti-cancer effects. Treatment with disulfiram or diethyldithiocarbamate has been shown in a mouse model to potentiate the immune response, reduce the incidence of autoimmune disease and activate a cytotoxic response against tumors. However, the exact mechanisms of these effects are not yet known.

The main focus of this work is to investigate the effects of the disulfiram metabolite CuET on immune cells and on possible changes in antigen presentation by the tumor cells themselves. Furthermore, the effect of CuET on the influence of phosphotyrosine-based signaling cascades was investigated.

In this thesis, I will first summarize the functions of the immune system, introduce cancer as a disease, the immune mechanisms involved in cancer cell degradation, and last but not least, disulfiram, its metabolite CuET and its investigated effects. Finally, the effect of the disulfiram metabolite CuET on cytotoxicity and tyrosine phosphorylation will be experimentally tested.

## 2    LITERATURE REVIEW

## 2.1   Immune system

The immune system is an organization of cells and molecules that have specialized functions in the defense of an organism. Cellular components of the immune system cooperate symbiotically to generate protective immune responses to ensure good health. An imbalanced immune system can lead to autoimmune diseases, cancer or chronic inflammation. Because of its memory, the immune system can remember and recognize pathogens years and decades after the first exposure. It gives the host long-term protection from reinfection with the same type of pathogen. The immune system can also distinguish between its cells and those of another organism and decide how and whether to respond to specific pathogens. Most of these mechanisms originated in evolutionary history as markers that allow cells to recognize and interact with each other to form symbiotic relationships. The simple method of molecular recognition has evolved into a highly complex immune system whose main feature is the ability of protein molecules to recognize and specifically bind to a particular shape structure on another molecule (Delves & Roitt, 2000; Benjamini et al., 2015).

There are two different types of immune responses that differ in response speed and specificity. These are named the innate and the adaptive responses. The main difference between these two types of responses is that innate (natural) responses occur to the same extent. However, the infectious agent is often encountered, whereas adaptive responses improve on repeated exposure to the infection (Delves & Roitt, 2000). The most significant differences between the innate and adaptive immune systems can are described in Table 1. The cells involved in the adaptive and innate immune response are shown in Figure 1.

*Table 1: Characteristics of the innate and adaptive immune response*

| CHARACTERISTICS | Innate immunity | Adaptive immunity |
|---|---|---|
| Response | Rapid (minutes – hours) | Slow (days) |
| Specificity | Antigen nonspecific | Antigen specific |
| Presence | Something already present in the body. | Created in response to exposure to a foreign substance. |
| Cells involved | Dendrictic cells (leukocyte), NK cells, macrophages.. | Cytotoxic CD8+ T cells, Helper CD4+ T cells, B cells, antigen-presenting cells.. |
| Immunological memory | None | Confer immunological memory |
| Immune response | Inflammation, complement-mediated killing, phagocytosis | Antibodies generation, microbial destruction by CD4+ and CD8+ T cells |
| Used against | Microbes | Microbes and non-microbial substances – antigens |
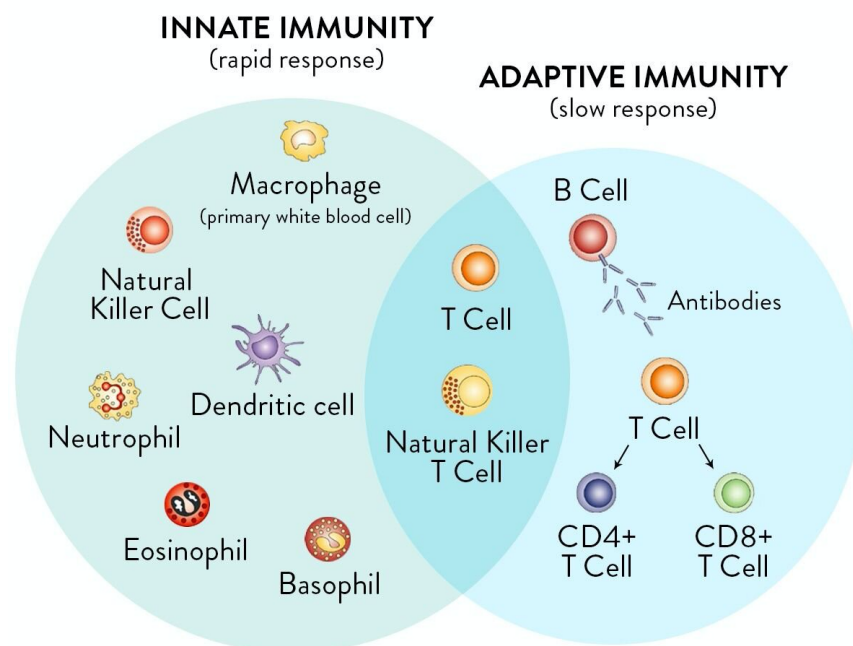


*Figure 1: Cells involved in the innate and adaptive immune response (MicrobeNotes.com)*

### 2.1.1 Innate immunity

Innate immunity is a primordial immune defense system that is present from birth. The primary function of this defense system is to provide a rapid first line of defense against pathogens that commonly infect humans (Benjamini et al., 2015). Innate immune response mechanisms can destroy or limit the spread of infection early, allowing time for the adaptive immune response to develop (Nicholson, 2016).

The innate immune system includes body surfaces and internal components such as skin, the mucous membranes, and the cough reflex that serve as barriers to environmental agents. Chemical influences, such as pH, secreted hydrolytic enzymes, and fatty acids also serve as barriers against invasion. Other elements of the innate immune system include noncellular components, complement and defensins (Benjamini et al., 2015).

The cells of innate immunity are polymorphonuclear leukocytes, dendritic cells, natural killer (T) cells, innate lymphoid cells, and phagocytic cells (granulocytes, macrophages (derived from blood monocytes), microglial cells of the central nervous system), which destroy and eliminate foreign material that has penetrated the physical and chemical barriers. Innate immune cells use germline-encoded receptors to detect and respond to microbes and other foreign antigens (Benjamini et al., 2015; Sompayrac, 2019).

### 2.1.2 Adaptive immunity

Adaptive immunity is a more specialized form of immunity, which is found only invertebrates. The cellular component of adaptive immunity consists of T-lymphocytes, B-lymphocytes, and plasma cells. Antigen-specific responses of these cells are the basis for this type of immunity. The adaptive response is very precise but takes several days or weeks to develop. The adaptive response has memory so subsequent exposure leads to a stronger and faster response (Delves & Roitt, 2000). One of the hallmarks of this defense system is that it can adapt to protect us against specific invaders (Nicholson, 2016).

If innate immune mechanisms do not eliminate infectious organisms, adaptive immune responses are triggered. The antigen is the compound to which the adaptive immune response is induced. An antigen is a small, specific molecule on a particular pathogen stimulating a response. Immune cells of the adaptive immune system can recognize and respond to a particular antigen or a few very similar ones. The first contact with a particular foreign substance that has penetrated the body starts a chain of events that induces an immune response with specificity against that foreign substance. These reactions result in the production of effector cells (antigen-specific lymphocytes) and

memory cells, which can later prevent reinfection by the same organism (Benjamini, 2015; Nicholson, 2016).

The effector cells responsible for adaptive immune responses are B and T cells arising from lymphocytes, a type of white blood cell produced together with other blood cells in the bone marrow. B and T cells are very similar, but there are differences between them. B cells arise from the maturation of immature lymphocytes in the bone marrow; T cells, on the other hand, travel to the thymus and mature there (Benjamini, 2015; Nicholson, 2016).

Each B or T cell develops unique surface proteins - specific receptors (not encoded in the germ line) during the maturation process. B cells develop antigen-specific B cell receptors (BCRs), and similarly, T cells develop antigen-specific T cell receptors (TCRs).

These surface proteins (receptors) recognize only a set of particular molecules on antigens. Therefore, each B and T cell recognizes very few molecules, but the entire population of human lymphocytes together should identify molecules from most pathogens. In an adaptive immune response, activated T and B cells with surface binding sites specific for molecules of a particular antigen will significantly increase their numbers and attack the pathogen (Benjamini, 2015; Malmquist & Prescott, 2018).

Another difference between B and T cells is that B cells target pathogens in the blood and lymph, mediating humoral immune responses, mainly by secreting antibodies. In contrast, T cells target infected cells in the body and participate in cell-mediated immune responses. There are three main types of T cells – killer T cells (cytotoxic T cells), helper T cells and regulatory T cells. Cytotoxic T cells destroy the body's own infected or unhealthy cells. Helper T cells secrete molecules that have a key effect on other cells' function in the immune response. The role of a third type of cell, regulatory T cells, is to prevent the immune system from overreacting or reacting inappropriately (Malmquist & Prescott, 2018; Sompayrac, 2019).

Because B and T cells are very potent weapons, they must be activated before they can function.

The first step is for the cell to encounter the antigen by specifically binding to it using specialized membrane proteins. It is this binding that triggers changes in immune cell activity - activation. In general, activation in these cells induces changes in gene expression and initiation of cell division. The next step is for the immune cells to attack the invading pathogens or infected cells. Finally, long-lived, pre-activated immune cells are generated in the memory phase of the adaptive immune response. These cells, memory

cells, are identical to the original cells that first encountered the pathogen, except that they have already undergone the activation step (Malmquist & Prescott, 2018; Sompayrac, 2019).

**B cells: Humolar immune response**

The humoral immune response involves the production of antibodies by B cells. B cells respond to encounters with an antigen by producing specific antibodies, circulating throughout the organism, and binding to the antigen. Each B cell has only one type of antigen receptor; this distinguishes them from each other. When a particular B cell encounters a specific antigen that binds to its receptor, the antigen molecule enters the cell, is processed by the cell, and presented on its surface - B cell activation. Subsequently, the activation causes the cell to divide rapidly, resulting in thousands of identical cells. The resulting cells function either as memory cells that remain inactive or as plasma cells that produce large amounts of antibodies. The antibodies produced by the B cell circulate through the bloodstream and lymphatic system and bind specific antigens, for example marking a pathogen for destruction by the phagocytosing cells of the innate immune system (Benjamini, 2015; Malmquist & Prescott, 2018).

**Cell-mediated immunity: T cells**

Even the activation of T cells begins when they encounter an antigen and bind to it with specific proteins on their surface. As with B cells, TCR proteins can only bind to one or a few similar antigens. Unlike B cells, T cells need the help of the innate immune system cells, known as antigen-presenting cells (APCs), to recognize pathogens. APCs include dendritic cells, macrophages, and B cells. APCs are cells that can process a protein antigen, break it down into peptides and present it in association with MHC molecules on the cell surface, where it can interact with the appropriate TCR (Benjamini, 2015; Malmquist & Prescott, 2018).

An essential system for antigen recognition by T cells is the APCs major histocompatibility complex (MHC), a genetic system responsible for distinguishing self from foreign antigens. Its primary function is to present antigens or fragments predominantly to T cells. In this case, the most crucial gene products of the MHC system are MHC class II molecules. MHC class II molecules are a class of MHC molecules usually found only on antigen-presenting cells. The whole process is based on phagocytosis of the APC, which engulfs the antigen; inside the cell, the antigen reacts with MHC molecules. Subsequently, the complex of antigen and molecules travels to the

cell surface, at which stage the antigen is recognizable to the T cell (Malmquist & Prescott, 2018).

T cells that bind their specific receptors to antigens presented on APCs are considered activated in this process. After activation, the cell divides, producing several thousand identical clones (Benjamini et al., 2015).

As mentioned, there are three main types of T cells. Helper T cells (CD4 T cells) attack pathogens by producing cytokinin's. Thanks to cytokines, the activity of innate immune cells – macrophages, cytotoxic T cells (CD8 T cells) and B cells - is greatly enhanced. In turn, cytotoxic T cells attack and destroy infected cells directly. Cytotoxic cells are crucial in protecting against viral infections because viruses replicate inside cells where they are protected from circulating antibodies. The resulting active cytotoxic cells identify infected host cells, bind to them, and kill them. Subsequently, the phagocytosing cells of the innate immune system clear the cellular debris and engulf and destroy any pathogens inside the cells. The cytotoxic T cells try to identify and eliminate the infected cells before the pathogen can multiply and escape, thus stopping the progression of the infection (Malmquist & Prescott, 2018).

### 2.1.3   Phosphotyrosine-based signal transduction

Phosphorylation of tyrosine in the protein structure produces phosphotyrosine from tyrosine. Chemically, it is the marking of tyrosine residues (on the hydroxyl group) with a phosphate group via protein kinases. Phosphorylation of tyrosine affects a particular protein's activity (function) and is considered a critical factor in cellular signal transduction and regulation of enzymatic activity. Three main functions mediate phosphotyrosine-based signal transduction: phosphorylation of specific target tyrosine residues (tyrosine kinase, TyrK), phosphate removal (phosphotyrosine phosphatase, PTP) and recognition of phosphorylated tyrosine residues (Src Homology 2, SH2). Phosphotyrosine can be detected using a specific antibody (Lim & Pawson, 2010; Cerulli & Kritzer, 2020). Signal transduction by the phosphorylation cascade is shown in Figure 2.
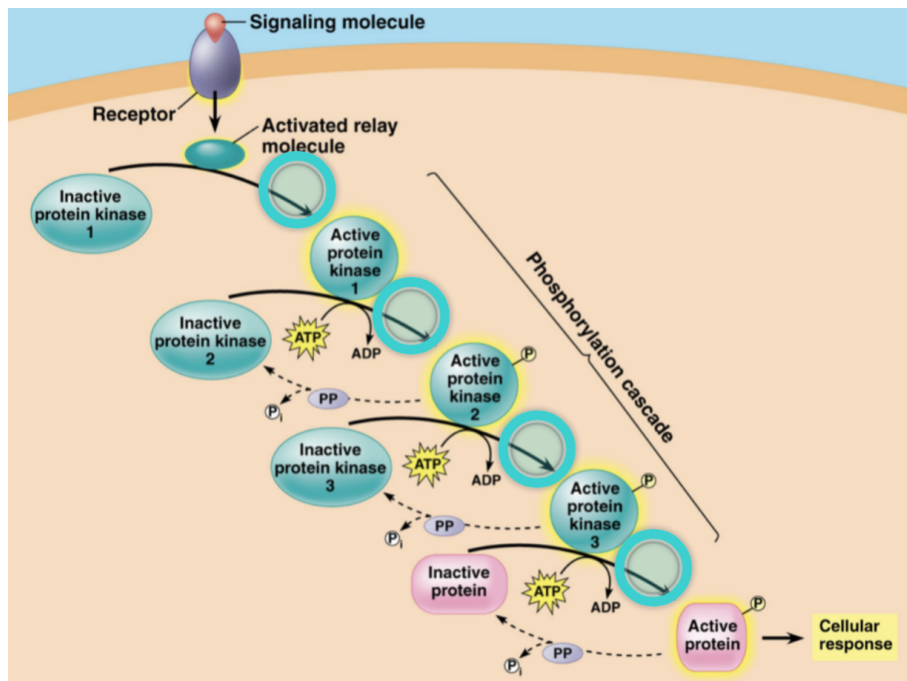


*Figure 2: Signal transduction by phosphorylation cascade*

## 2.2 Cancer

Cancer is a complex disease, in which genetic and epigenetic modifications have been implicated in its development, is the second leading cause of death worldwide. It's a group of diseases in which cell divide continuously and excessively. A problem in the control system and dysregulation of cellular pathways involved in proliferation, cell cycle, apoptosis, cell metabolism, migration, differentiation etc., usually causes cancer. The disease develops when several control systems are damaged in one cell. In cancer, two central systems can be damaged: systems that promote cell growth (proliferation) and safety systems that protect against "irresponsible" cell growth. The growth-promoting systems in cells usually work well. However, if one of these systems fails and starts to proliferate excessively, this is a precondition for the cell to become cancerous. These systems are made up of proteins, so malfunctions in their function are caused mainly by changes in gene expression, usually due to mutations. A gene that can cause inappropriate cell proliferation (after mutation) is called a proto-oncogene (Sompayrac, 2019; Galon & Brunis, 2020; Derakhshani et al., 2021).

Our cells are equipped with internal protective systems that are responsible for protecting against malfunctions in the control systems that promote cell proliferation. These protective systems are designed to prevent mutations in the first place and to deal with mutations once they occur. Cells have DNA repair systems that repair damaged DNA to protect the cell from mutations. Sometimes, however, the DNA repair systems miss a mutation in the DNA, and then the second protective system comes in and monitors for unrepaired mutations. If the genetic damage is severe, the protection system triggers the cell to commit suicide, eliminating the possibility of it becoming a cancer cell. One of the most important components of this secondary protection system is the p53 protein (encoded by tumor suppressor genes), which protects the cell from growing uncontrollably. Mutations in the gene for the p53 protein have been found in most human cancers (Sompayrac, 2019; Matthews et al., 2022).

### 2.2.1 Cancer cells

As mentioned, cancer is caused by increased cell proliferation, resistance to apoptosis, genetic instability, and angiogenesis, reprogramming of metabolism and cell migration. Most of these changes are caused by dysregulation of signaling pathways by altering the activity of cellular regulators. Cancer cells behave differently from normal cells in the body mainly in the process of cell division. For example, cancer cells may make their

own growth factors or trick adjoining cells into producing growth factors to support them. Cancer cells also ignore signals that would cause a normal cell to stop dividing. Another characteristic is that these cells can divide many more times than normal body cells - "replicative immortality". The replicative immortality of cancer cells is because telomerase activity does not decrease, maintaining cell proliferation. Cancer cells also differ from normal ones in properties unrelated to the cell cycle. For example, cells can migrate to other parts of the body – metastasize. Cancer cells also do not undergo programmed cell death, or apoptosis, under the same conditions as normal cells (Hanahan & Weinberg, 2011; Matthews et al., 2022).

Every cell has many mechanisms that repair damaged DNA, limiting cell division and thus preventing cancer. Most human cancers arise spontaneously when several mutations accumulate in a single cell, giving it the characteristics of a cancer cell. This means that the cells divide more rapidly due to such mutations, escape external and internal control of division, and avoid programmed cell death. These mutations can arise from errors in copying DNA or from exposure to mutagenic substances (carcinogens). These mutagens may be by-products of normal cellular metabolism or may be present in the air we breathe and the food we eat. Radiation (including UV radiation) can also cause mutations. Cancer cells can also be associated with viruses because some viruses produce proteins that can disrupt the functioning of protective systems. Infection with these special cancer viruses reduces the total number of cellular genes that must be mutated to turn a normal cell into a cancer cell, and infection with these cancer viruses therefore accelerates the development of cancer (Hanahan & Weinberg, 2011; Sompayrac, 2019).

Cancer cells arise due to mutations in mainly two types of regulators – over-activation of positive regulators (to become oncogenic) and inactivation of negative regulators (suppressors). Genes that normally block cell cycle progression are referred to as tumor suppressors. For example, a protein that stops cell cycle progression in response to DNA damage stops triggering the reaction. One of the most important tumor suppressors is the before mentioned tumor protein p53, which has a key function in the response to DNA damage (Hanahan & Weinberg, 2011; Sompayrac, 2019).

It is believed that cancer cells form during life and are destroyed by immune effector mechanisms. However, these mechanisms are not always successful (Benjamini et al., 2015). The characteristic differences between a normal cell and a tumor cell are shown in Table 2.

*Table 2: Characteristic differences between a normal cell and a tumor cell*

| NORMAL CELL | CANCER CELL |
|---|---|
| Single nuclus | Multiple nuclei |
| Single nucleolus | Multiple nucleoli |
| Spherical shape of a nucleus | Irregular shape of a nucleus |
| Large cytoplasm | Small cytoplasm |
| Fine chromatin | Coarse chromatin |
| Unfixable DNA damage → apoptosis | Unfixable DNA damage → cell continues dividing |
| Uniform shape and size of cells and nuclei | Variations in size and shapes of cells and nuclei |

### 2.2.2 Tumor antigens

Tumor antigens are substances usually of a protein nature expressed by cancer cells. They are an important part of antitumor immunity and its mechanisms. The existence of these antigens and their presence on the MHC allows the immune system to recognize cancer cells and subsequently respond to them. To be able to induce an immune response, antigens must have the following properties – foreignness, high molecular weight, chemical complexity, and degradability with the ability to present on the host MHC. Antigens are presented on MHC cell surface molecules where they are recognized by receptors on immune cells. There are two main types of tumor antigens, tumor-associated antigens (TAA) and tumor-specific antigens (TSA). TAAs are antigens that are expressed by both cancer cells and other cells. The difference between tumor and healthy cells is the level of expression. TSAs are specific to cancer cells and are not found on normal healthy cells (Benjamini et al., 2015; Feola et al., 2020). The differences between the various types of antigens are shown in Figure 3.

Self-antigens
expression in healthy tissues

TAA
overexpression of self-antigen

TSA
neoantigens, own antigens

Cancer cell
Health cell
Antigens
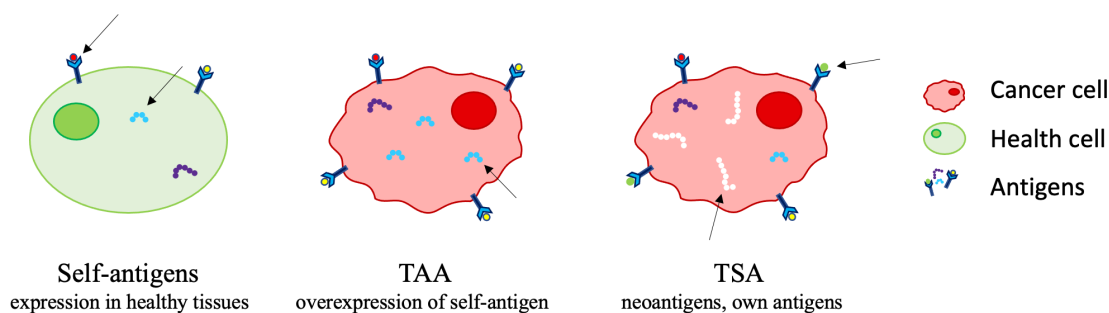
*Figure 3: Types of antigens expressed by tumour cells*

### 2.2.3 Role of copper in cancer progression

Copper is an essential micronutrient for the development and replication of all eukaryotes. Because copper cannot be created or destroyed by metabolic processes, it must be obtained from external sources. Copper enzymes are involved in a wide range of metabolic processes, including aerobic respiration, pigmentation, peptide amination, catecholamine biosynthesis, superoxide dismutation, and extra cell matrix biosynthesis. In increased amounts, free copper is potentially toxic - it inhibits the function of oxidative cysteine enzymes or reacts with hydrogen peroxide to form a hydroxyl radical. Under normal conditions, however, copper transporters and proteins prevent the formation of the free copper ion in the cytoplasm, because the transport and distribution of copper is very tightly controlled by several cellular mechanisms (Shanbhag et al., 2021).

As a cofactor regulating the activity of many enzymes and structural proteins in cancer cells, it plays a key role in cancer progression. Many studies have shown that copper levels (in serum and tumor tissues) are adapted to cancer patients. The mechanism of copper in tumor progression is based primarily on its ability to elicit redox reactions. In the intracellular environment, they contain reactive oxygen species (ROS), which activate a prooncogenic signaling mechanism different for tumor cells and proliferation. Elevated copper levels also modulate signaling pathways mediating the escape of tumor cells from the immune system (Kannappan et al., 2021; Ge et al., 2022).

Specific functions of copper in mitogenic signaling pathways controlling both normal development and oncogenesis have also been detected. Copper uptake stimulates the mitogen-activated protein kinase MAPK (MAPK-ERK) pathway, which is involved in a wide range of normal biological processes (proliferation, differentiation, and motility) that are regulated by the interaction of growth factors with receptor tyrosine kinases. The kinase in this pathway, MEK1, contains a high affinity copper binding site that, when

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bound to copper, stimulates MEK1-dependent phosphorylation of ERK1/2 (Shanbhag et al., 2021).

However, copper is also a nutrient that limits the rate at which cancer cells grow and multiply. Copper chelators used with food have been shown to have antineoplastic and antimetastatic effects. This is because cancer cells have higher demands on copper compared to non-dividing cells, which can be used for treatment with copper chelating agents to suppress tumor growth and travel (Shanbhag et al., 2021).

### 2.2.4 Cancer and signaling pathways

As mentioned, dysregulation of various signaling pathways involved in proliferation, survival, metabolism, migration, differentiation are involved in the development of cancer. The signal in most of these pathways is transmitted through phosphorylation (Derakhshani et al., 2021).

Elimination of tumor cells by targeting signaling pathways is a complicated mechanism. However, intertumoral heterogeneity and genetic instability of tumor cells can lead to the formation of various neoantigens, which will ultimately allow the integration of lymphocytes into the tumor microenvironment. Overexpression of oncogenes and oncoproteins may promote abnormal signals that lead to tumorigenesis. As already mentioned, tumorigenesis is associated with the influence of two types of genes - proto-oncogene activation and suppressor gene inactivation. Oncogenic mutations can then affect the nuclear targets of signaling pathways. One of the most frequently signaling pathways among all tumor types is the Receptor Tyrosine Kinase (RTK)-Ras-Extracellular signal-regulated kinase (ERK) signaling pathway. Dysregulated cell proliferation is also essential for cancer development. Altered expression and activation of cell cycle dependent proteins are the main culprits of dysregulated tumor proliferation. Because of the high rates of mutations and intertumoral heterogeneity in tumor cells, targeting molecules and genes involved in signaling pathways cannot eliminate mutating cancer cells. However, genetic instability of tumor cells can lead to the formation of tumor neoantigens, which are subsequently recognized by immune cells. Targeting any of the inhibitory immunity checkpoints (such as PD-1) is one approach to stimulating anti-tumor immune responses. Block blocking increases CD8 infiltration into the tumor (Derakhshani et al., 2021). Immune checkpoints involved in CD8 cell dysfunction are mentioned in Figure 4.
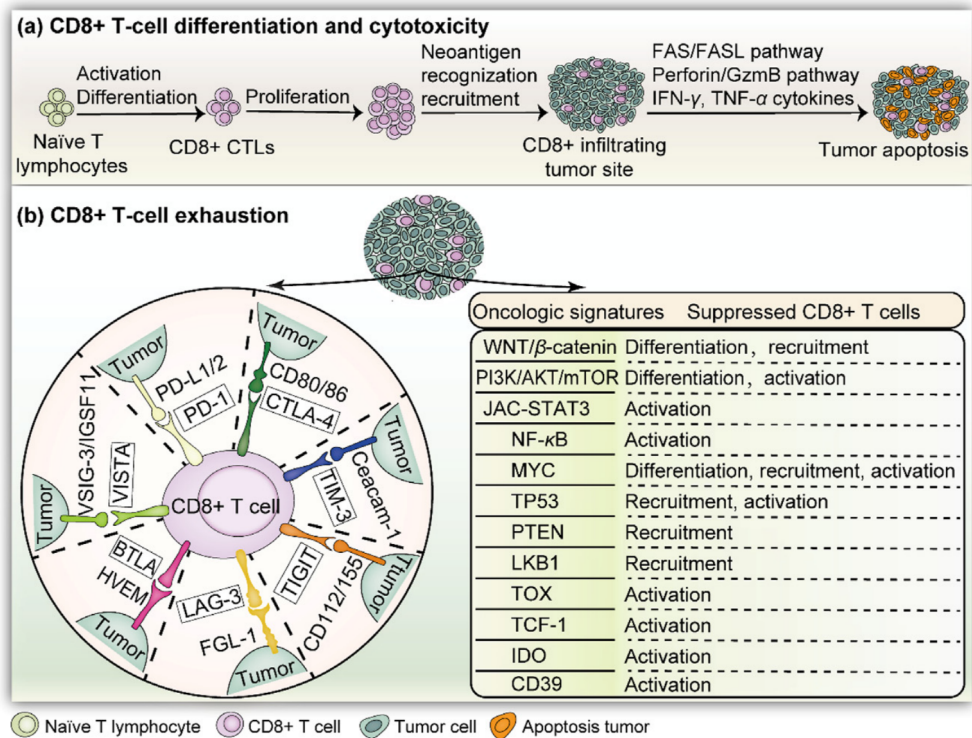
**(a) CD8+ T-cell differentiation and cytotoxicity**

Activation Differentiation → Proliferation → Neoantigen recognization recruitment → CD8+ infiltrating tumor site → FAS/FASL pathway Perforin/GzmB pathway IFN-γ, TNF-α cytokines → Tumor apoptosis

Naïve T lymphocytes    CD8+ CTLs

**(b) CD8+ T-cell exhaustion**

Tumor — PD-L1/2 — PD-1
VSIG-3/IGSF11 — VISTA
Tumor — CD80/86 — CTLA-4
Ceacam-1 — TIM-3
BTLA — HVEM
LAG-3 — TIGIT
FGL-1 — CD112/155
CD8+ T cell

| Oncologic signatures | Suppressed CD8+ T cells |
|---|---|
| WNT/β-catenin | Differentiation, recruitment |
| PI3K/AKT/mTOR | Differentiation, activation |
| JAC-STAT3 | Activation |
| NF-κB | Activation |
| MYC | Differentiation, recruitment, activation |
| TP53 | Recruitment, activation |
| PTEN | Recruitment |
| LKB1 | Recruitment |
| TOX | Activation |
| TCF-1 | Activation |
| IDO | Activation |
| CD39 | Activation |

○ Naïve T lymphocyte  ○ CD8+ T cell  ○ Tumor cell  ○ Apoptosis tumor

*Figure 4: A) Infiltration of CD8 cells into the tumor – differentiation, cytotoxicity. B) Immune checkpoints involved in CD8 cell dysfunction*

### 2.2.5 Immune system in cancer cells destruction

The goal of the immune response in cancer is to eliminate cancer cells. The main players in this process are cytotoxic T cells and natural killer cells. Immune effector mechanisms that contribute to the destruction of cancer cells are summarized in Table 3. Immune responses against cancer cells arise in response to the expression of cell surface components on cancer cells that are not present on normal cells and are recognized as antigenic molecules. The extent of the immune response against a cancer cell depends on the mode of antigen presentation (El-Sharkawy, 2014).

The fight against cancer cells involves adaptive immunity, both humoral and cellular. The humoral part is represented by B cells that produce antibodies, and the cellular part by T cells. Other functional cells in reactions to cancer cells are dendritic cells and APCs. APCs are activated during an innate immune response, receive antigen, and present it to effector cells of the adaptive system – T cells. T cells are responsible for recognizing antigens presented by APC by TCR and the subsequent immune response. Other effector cells are natural killer (NK) cells, activated during the innate immune response, destroying the tumor by killer activating receptors expressed on their surface (El-Sharkawy, 2014). Effector mechanisms in tumor cell destruction are summarized in Table 3.

*Table 3: Effector mechanisms of the immune system in tumor cell destruction*

| EFFECTOR MECHANISM | COMMENTS |
|---|---|
| Antibodies, B cells (complement-mediated lysis, opsonization) | Role in tumor immunity poorly understood, helper of other cells |
| T cells (cytolysis, apoptosis) | Rejection of virally and chemically induced tumors |
| NK cells (cytolysis, apoptosis) | Tumor cells not expressing one of the MHC classes I alleles are effectively rejected by NK cells |
| LAK cells (cytolysis, apoptosis) | Antitumor responses seen in certain human cancers following adoptive transfer of LAK cells |
| Macrophages, neutrophils (cytostasis, cytolysis, phagocytosis) | Can be activated by bacterial products to destroy or inhibit tumor cell growth |
| Cytokines (apoptosis, recruitment of inflammatory cell) | Growth inhibition can occur using adoptively transferred tumor cells transfected with certain cytokines |

### 2.2.6  T lymphocytes in the anti-tumor response

T-lymphocytes, predominantly cytotoxic, are a major component of beneficial anti-tumor immunity and provide a direct response to target cells by killing tumor cells and destroying the tumor (El-Sharkawy, 2014). Figure 4 shows the response of a T lymphocyte to an interaction with a tumor antigen.

T cells are initially inactive and programmed to mature T cells in the team. As mentioned, TCRs are essential for the realization of molecular binding and receptor recognition. T lymphocytes express CD4 and CD8 protein subtypes after their differentiation, development and maturation.CD4 lymphocytes (helper T cells) play an important regulatory role in anti-tumor effect. CD4 molecules are involved in the activation of antigen recognition by TCR and in enhancing the sensitive interaction between APCs and T cells through binding to MHC. CD4 also produce cytokines that promote macrophage-mediated immunity and activate B cells to produce antibodies. CH4

can also enhance the anti-tumor response of CD8 cells by triggering their gene program to enhance cytotoxic T cell function (Xie et al., 2021).

CD8s are the main killer immune cells. During T cell signal activation, TCRs bind to antigenic peptides in MHC I molecules and initiate signal activation. This process is promoted by the binding of the CD8 coreceptor to the domain of MHC molecules, which amplifies the signal transmission. Once activated, T cells begin to proliferate and differentiate into CD8 effector cells. When CD8 cells overcome immunosuppressive mechanisms and penetrate between tumor cells, they initiate a cytotoxic response and cell elimination. CD8 cells inhibit tumor proliferation and disrupt metastasis upon recognition of intracellular antigens. Upon recognition of a specific antigen, unique cytolytic mediators are generated by active cytotoxic CD8, which elicit an effective response against target tumors. In the host, T cells are programmed to differentiate into cytotoxic CD8 and transported to the tumor site to destroy the tumor cells (Xie et al., 2021). However, there are some limitations, mentioned in Chapter 2.2.8. T cell response to APC presented antigen is shown in Figure 5.
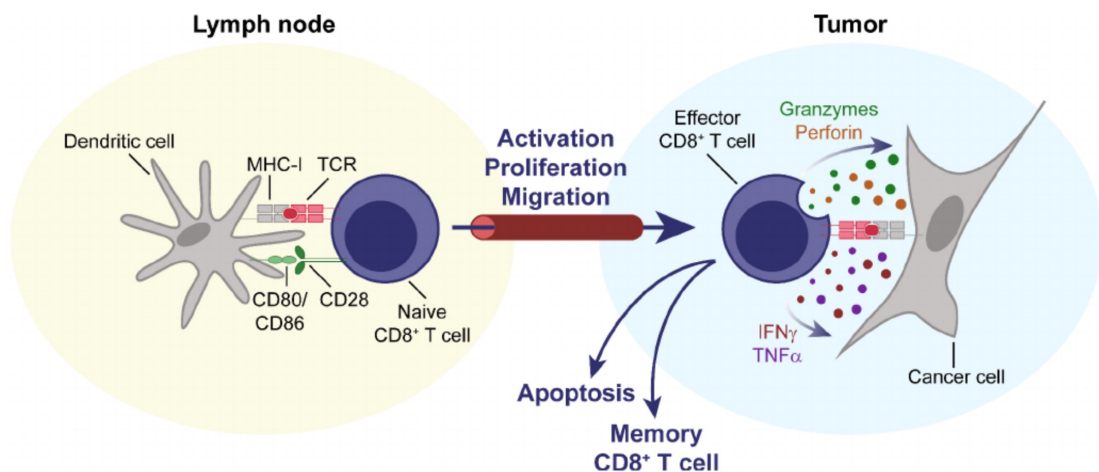


*Figure 5: CD8+ T cells are key effectors of the anti-tumor immune response (Drijvers et al., 2020)*

### 2.2.7 Other immune cells in antitumor response

The function of B cells in responding to cancer cells is also very important, although their role in this regard has been underestimated until recently. Activated B cells can either present tumor-associated antigens to T cells or produce antibodies that enhance antigen presentation to T cells or directly kill cancer cells. Antibodies produced by B cells can also increase macrophage activity. Destructive properties for cancer cells have been demonstrated for IgG and IgM antibodies. Thus, in general, B cells are essential for the establishment of the T cell immune response (Fridman et al., 2021).

Dendritic cells (DCs) have the APCs function and they are activated during the innate immune response. Its main function is to present antigen to effector cells of the adaptive immune system – CD4+ and CD8+ T cells. Antigens released by necrotic, dying, or apoptotic tumors are taken up by DCs and represented to T cells. These dendritic cells and other APCs are responsible for T cells activation (Benjamini et al., 2015).

NK-lymphocytes are also responsible for killing tumor cells and destroying tumors. NK cells are a lymphoid population of peripheral blood mononuclear cells that are capable of lysing tumor and virus-infected cells. NK cells are activated during the innate response, are not antigen specific, but kill tumors through killer activating receptors (KARs) (El-Sharkawy, 2014; Benjamini et al., 2015; Martínez-Lostao et al., 2015).

Other effector cells are macrophages, which upon activation can cause selective cytostasis or lysis of tumor cells by lysosomal enzymes. The main helper of macrophages are helper T-lymphocytes, which upon activation by secretion of cytokinin, attract macrophages to the antigenic area (Benjamini et al., 2015).

## 2.2.8 Limitations of the effectiveness of the immune response against tumors

The above shows that an immune response against tumor cells can be induced. However, several mechanisms may operate in tumor cells that allow further tumor growth in the host. The escape of tumor cells from destruction by the immune system is influenced by factors related to either the tumor or the host. Tumor-related factors include those related to defective immunosensitivity – lack of antigenic epitope, antigenic masking of the tumor (appearing as a normal cell), failure to provide a suitable antigenic target, production of inhibitory substances by the tumor (for example cytokines). Host-related factors include immunosuppression, defects in T cell signaling, inadequate APC antigen presentation, and co on (Benjamini et al., 2015).

However, tumor cells are limited by fewer CD8 immunogenic features due to internal heterogeneity. CD8 activity can also be inhibited by stromal components between tumor cells. Tumor cells are able to induce a state associated with low immunogenic activity in CD8 cells, leading to tumor resistance, which is a major obstacle to treatment. The ratio of recovering T cells is regulated by the tumor primarily through the activation of checkpoint inhibitory pathways such as programmed cell death 1 (PD-1). Immune checkpoints are responsible for tumor-specific depletion of CD8 cells limiting their cytotoxic effect. CD8 cells may be dysfunctional due to a lack of cytolytic factors or just by elevated checkpoint levels. As mentioned, the PD1 causes suppression of CD8 stimulation by binding the ligand PD-L1/2, which is abundant in tumor tissues. The

resulting PD1-PDL1 complex promotes tumor escape from the immune system and inhibits the immune response of antigen-specific CD8 (Xie et al., 2021).

Anti-tumor immune attacks can also target other immune populations such as macrophages, dendritic cells and regulatory T cells (Xie et al., 2021).

## 2.3 Disulfiram and its metabolite – diethyldithiocarbamate

Disulfiram (DSF; known by the trade name Antabuse) is a symmetrical disulfide - thiuram derivative that was developed to treat alcoholism, it has been used to treat alcoholism for over 70 years. DSF has been found to have antitumor activity in various types of cancer. Therefore, because the safety profile of DSF and its pharmacokinetics is very well studied, repurposing DSF as a new anticancer drug is a promising strategy. At physiological pH, DSF is 99% metabolized (by ionization) to diethyldithiocarbamate in the body. The advantages of DSF for cancer treatment include its specific mechanisms of action such as induction of reactive oxygen species (ROS) accumulation, targeting of aldehyde dehydrogenase, immunostimulatory properties, its ability to target even cancer stem cells, and sensitization of cancer cells to radiotherapy. DSF also chelates heavy metals very well, forming complexes with copper, which enhances its antitumor activity (Gessner & Gessner, 1992; H. Li et al., 2020).

### 2.3.1 Physical and chemical properties of DFS and its metabolite diethyldithiocarbamate

Chemically, disulfiram is tetraethylthiuram disulfide or tetraethylthioperoxydicarbon-diamide. It is a dimeric molecule in which the two diethyl-dithiocarbamate molecules are linked via sulfur atoms by disulfide bonds. The chemical formula is shown in Figure 6. DSF is relatively insoluble in water, and saturation occurs at approximately 40-100 μM. To obtain solutions with more than 50 μM, it is necessary to use a transporter. The disulfide bond is relatively unstable and by heating the compound can be dissociated into two dithiocarbamate radicals. In solution, DSF is readily reduced to diethyldithiocarbamate (DSH) by ascorbic acid and compounds with various sulfhydryl's. DSF can also be reduced by copper ions, resulting in forming a copper ion complex. DSH can also be readily reversibly oxidized to DSF by, for example, cytochrome c or hydrogen peroxide (Gessner & Gessner, 1992).



*Figure 6: Chemical structure of disulfiram and its reduced form diethyldithiocarbamate (Gessner & Gessner, 1992).*

### 2.3.2    Metabolism of DFS – formation of copper complexes

Disulfiram (DSF) and diethyldithiocarbamate (DSH) interact with blood components primarily in two ways. After ingestion, DSF interacts with plasma copper and is very rapidly converted to the bis(diethyldithiocarbamate)copper complex. In the absence of metal ions, DSF may also react with proteins to form mixed disulfides (Gessner & Gessner, 1992).

The first step in DSF metabolism is reducing the disulfide bond (using endogenous thiols, sulfhydryl groups of proteins or reduced forms of metal ions. The reaction results in the formation of diethyldithiocarbamic acid. Sulfhydryl groups of thiols and proteins react with DSF to immediately form one equivalent of DSH. A second equivalent of DSH is formed by subsequent reduction of a mixed disulfide containing a DS residue. DSH can be readily oxidized back to DSF (using hydrogen peroxide or $Fe^{3+}$ (Gessner & Gessner, 1992; McMahon et al., 2020).

Copper complexes are formed due to the high affinity of DSF and DSH for heavy metal ions, predominantly copper. Disulfiram shows excellent efficacy precisely in combination with copper ions. The resulting copper diethyldithiocarbamate complex, which occurs naturally in the blood, is considered the main active anti-cancer component. However, administration of a pre-prepared complex shows better antitumour efficacy (McMahon et al., 2020). The formation of CuET is shown in Figure 7.
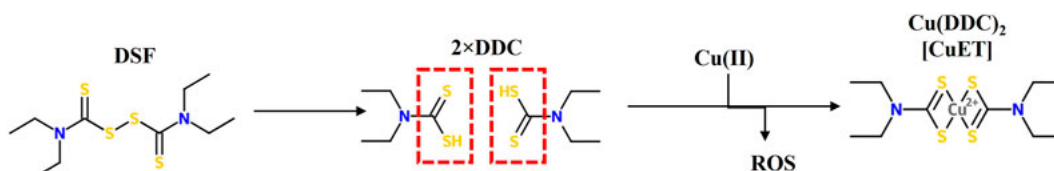


*Figure 7: Formation of CuET complex from disulfiram and copper (Kannappan et al., 2021)*

DSH is a ligand of heavy metals and forms complexes with them. The formation of binary complexes of heavy metals and DSH results in an increase in the solubility of metals in lipids, thus accelerating the passage of metals through lipid barriers. The most physiologically accessible of the metal anions to which DSF and DSH have high affinity is copper (Cu). DSF is stoichiometrically reduced and converted to the Cu(DS)2 complex (Gessner & Gessner, 1992; McMahon et al., 2020).

### 2.3.3    Immunomodulatory effects of disulfiram

DSH has many interesting and potentially important immunomodulatory effects. Immunostimulation is mediated indirectly through effects on T cells. The most common DSF-induced immunostimulatory effects include lymphoproliferative responses to

alloantigens, T cell cytotoxicity, antibody-dependent cellular cytotoxicity, promotion of phagocytosis, increased natural killer cell activity, and T cell differentiation (Gessner & Gessner, 1992; Y. Li et al., 2018).

### 2.3.4 DFS and cancer

DFS has been mentioned in recent years in the context of cancer treatment. Several studies have described promising treatment results after disulfiram treatment in various types of cancer. As mentioned, DSF is metabolized mainly to DSH and other metabolites in the body. DSH then forms complexes with the exchangeable ions to form bis(diethyldithiocarbamate) - CuET. This metabolite is considered to be the compound with the most significant anticancer potential. From this, it can be concluded that the antitumor activity of DSF is dependent on the presence of copper since copper potentiates its activity (Skrott et al., 2017; Wiser Drozdkova & Smesny Trtkova, 2021).

### 2.3.5 CuET

Copper (Cu) is a trace element and plays a vital role in the body. It is an important element in redox reactions and induces the production of reactive oxygen species. Chelating Cu ions increase the antitumor activity of DSF to produce bis(diethyldithiocarbamate)-copper (CuET) with more pronounced antitumor activity. By forming a complex with Cu, disulfiram improves copper transport into tumor cells. The concentration of copper in tumor cells then allows DSF to target tumor cells instead of normal cells (Carpentieri et al., 1986; Duan et al., 2014; Kannappan et al., 2021; Meng et al., 2021).

CuET itself exhibits cytotoxic effects or can function as an adjuvant by sensitizing tumor cells to many chemotherapeutic agents. The antitumor mechanisms of CuET itself have not yet been fully elucidated. The mechanism generally involves the induction of apoptosis in tumor cells. In addition, reactive oxygen species (ROS) can be generated by the chelation reaction, leading to damage of DNA, proteins, lipids, etc. in tumor cells, which also causes apoptosis. Formation of the CuET complex is therefore a more potent inducer of ROS. Another anti-cancer mechanism is the inhibition of the signaling pathway of the protein complex, nuclear factor kappa-B (NFkB). As a transcriptional regulator of various genes, NFkB has high activity in cancer cells and its high levels in tumor tissue are associated with drug resistance and blockade of apoptosis. It functions as a regulator of cell signaling pathways for survival, stem cell, resistance, engiogenesis and metastasis (Carpentieri et al., 1986; Duan et al., 2014; Kannappan et al., 2021; Meng et al., 2021).

Other functions of the CuET complex include suppression of the aforementioned STAT3 signalling, which then inhibits cell viability and induces apoptosis. In cells treated with the complex, STAT3 levels are significantly reduced. This means that apoptosis is probably caused by inhibition of STAT3 expression and phosphorylation (Kim et al., 2017; Y. Li et al., 2018; H. Li et al., 2020).

**CuET-induced Cell Death**

The basic anti-tumor mechanism is based on increased levels of copper in tumor cells. DSF penetrates tumor cells by forming a complex with copper; the elevated levels of copper in tumor cells allow DFS to target these cells rather than normal cells expressing low levels of copper. The formation of the complex leads to the generation of ROS that induce apoptosis in tumor cells. Another antitumor mechanism is inhibition of superoxide dismutase and cooperativity with glutathione reductase. Both of these enzymes are important for the cell to resist oxidative stress caused by ROS. ROS generated by the complex accumulate in the cells, this leads to massive DNA damage that induces mitochondrial pore opening and apoptosis via the MAPK trigger. ROS-induced apoptosis is dependent on sustained activation of the MAPK pathway, which phosphorylates mitochondrial proapoptotic proteins. Inhibitors of the MAPK pathway in ROS-induced apoptosis reduce the sytotoxic effect of the complex, suggesting its importance in apoptosis. The complex also reduces the expression of various genes involved in DNA repair pathways (Kannappan et al., 2021). The mechanism of anticancer activity of DSF/Cu is shown in Figure 8.
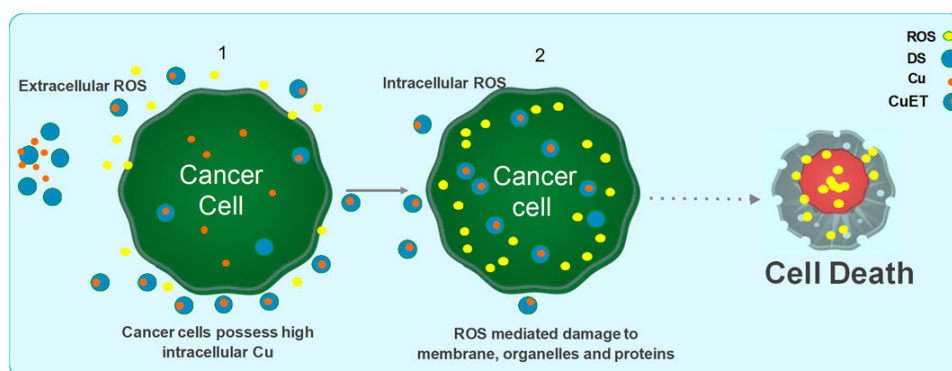


*Figure 8: Mechanism of action of DSF/Cu induced anticancer activity. DSF and Cu reaction generates extracellular ROS and damage the membrane proteins. (Kannappan et al., 2021)*

**CuET and proteasome inhibition**

The functional proteasome protein complex is important for the degradation of unnecessary or damaged proteins and maintenance of synchronized expression of proteins regulating cell cycle and apoptosis. The CuET complex has been shown to have

proteasome inhibitory activity. Inhibition of proteasome activity leads to accumulation of poly-ubiquitinated proteins and cytotoxic protein aggregates, resulting in inhibition of cell cycle progression and subsequent apoptosis. Proteasome activity is also significant for NF-kB function; if the CuET complex blocks the proteasome system, NF-kB is inhibited, preventing its nuclear translocation and transcriptional regulator function. This results in promoting apoptosis or sensitization of cancer cells to anticancer drugs (Skrott & Cvek, 2012; Skrott et al., 2017; Kannappan et al., 2021).

Inhibition of the proteasome is mediated by the p97-NPL4-UFDI pathway, which is localized upstream of the proteasome. The complex binds to NPL4 with high affinity because NPL4 contains two zinc-finger domains challenging divalent metals and metal complexes. Binding blocks the NPL4 conformational switch necessary for p97 unfolding activity, formation of protein aggregates, and deactivation of P97 segregase. This process results in the accumulation of misfolded proteins in cells and immobilization of the otherwise highly mobile NPL4-p97 complex. These events trigger a heat shock response and endoplasmic reticulum reaction to the misfolded protein, this eventually leads to cell death (Skrott & Cvek, 2012; Skrott et al., 2017; Kannappan et al., 2021).
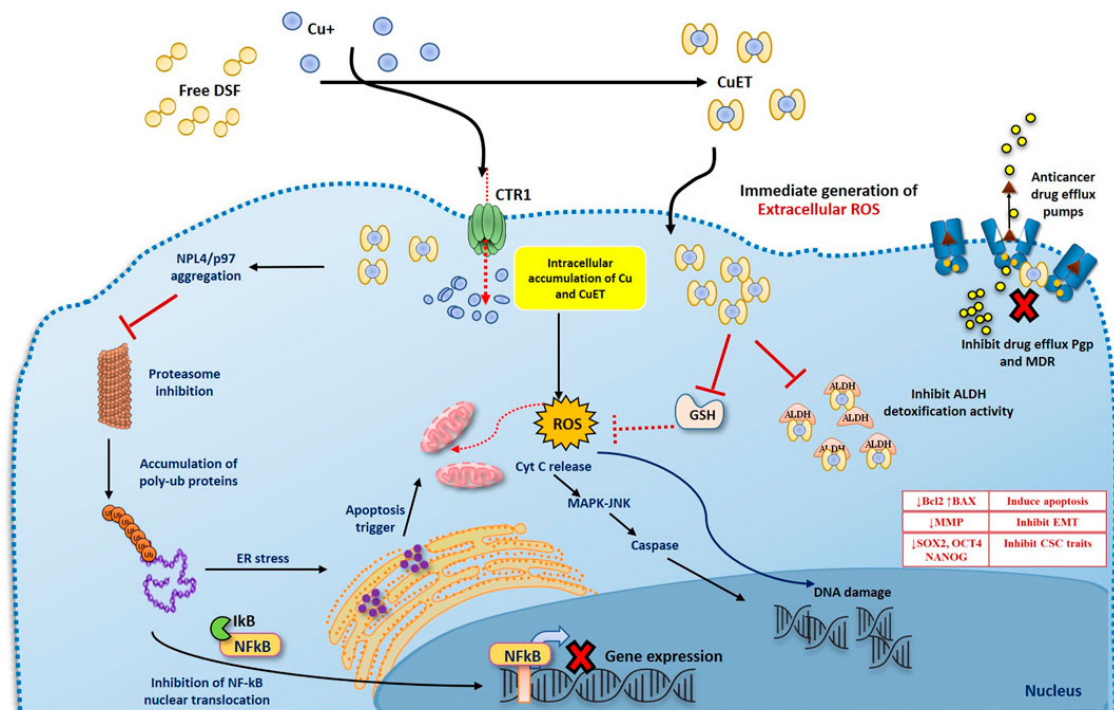


*Figure 9: Two phases anticancer model of DSS/DSH plus copper induced cancer cell death. 1. DSF or DSH and copper react with generation ROS which induce cancer cell apoptosis. 2. The reaction-generated compound, CuDDC2 (CuET), can easily penetrate cancer cells and trigger intracellular ROS generation and induce cancer cell apoptosis. (Kannappan et al., 2021)*

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### 2.3.6 Limitations of CuET effectivity

Although the CuET complex shows high toxicity in tumour cells in vitro, clinical studies using this complex have not shown positive data. Since the DSF+Cu reaction must occur near or inside the tumour cell, DSF is currently administered orally. The ineffectiveness may be due to the short half-life of the currently available oral version of DSF in the bloodstream (2-4 min). DSF is rapidly reduced to 2 molecules of DSH after ingestion; DSH accumulates in the liver, but is rapidly degraded there, losing the active functional thiol groups and the ability to chelate with metal ions (Kannappan et al., 2021).

# 3  MATERIALS AND METHODS

## 3.1  Biological material

The B16F10 mouse melanoma cell line used for experiments was obtained from ATCC (American Type Tissue Culture Collection). Cell line was cultivated in a Dulbecco`s modified Eagle`s medium (D-MEM). The incubation conditions of the cells were at 37 °C in humified 5% carbon dioxide atmosphere in a regular water jacked incubator Thermo Scientific. Cell line was passaged every 2-3 days to maintain exponential growth around 50 000 cells/ml. The passage limit was 15.

Splenocytes were obtained from spleen of C57BL/6 mice 40 week of age. Mice were bred and maintained in the laboratory animal facilities at IMTM in pathogen-free conditions, allowing free access to food and water.

## 3.2 Chemicals and reagents

- CuET - Diethyldithiocarbamate (Sigma-Aldrich, Cat. # D3506). Dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 2.5 mM and subsequently in complete medium to a concentration of 1 nM.
- DMSO (Sigma-Aldrich, Cat. # D4540 )
- Propidium iodide (Sigma-Aldrich, Cat. # 81845)
- Trypan blue (Sigma-Aldrich, Cat. # 93595)
- Ponceau S (Sigma-Aldrich, Cat. # P3504)
- Pierce$^{TM}$ BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. #23225
- Phosphotyrosine Monoclonal Antibody (pY20), FITC (Thermo Fisher Scientific, Cat. # MA1-12442)
- phospho-Tyrosine Monoclonal Antibody (Py20), eBioscience$^{TM}$ (ThermoFisher Scientific, Cat. # 14-5001-82), diluted in TBST a mixture of tris-buffered saline and Polysorbate 20 (1:1000)
- Secondary anti-mouse IgG horseradish peroxidase-conjugated antibody, diluted in dried milk with TBST (1:5000)
- ECL$^{TM}$ Western Blotting System
- Spectra$^{TM}$ Multicolor Broad Range Protein Ladder (Thermo Fisher
- Halt$^{TM}$ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Cat. # 78420)
- Halt$^{TM}$ Protease Inhibitor Cocktail (100X) (Thermo Fisher Scientific, Cat. # 87786)
- ECL$^{TM}$ Prime Western Blotting Detection Reagent (Sigma-Aldrich, Cat. # GERPN2236)

## 3.3 List of solutions

- 1x PBS: 10x PBS 80 g NaCl, 2 g KCl, 14,4 g Na$_2$HPO$_4 \cdot$2H$_2$O, 2 g KH$_2$PO$_4$, dissolved in 800 ml dH$_2$0 and added to 1 l; preparation: mix 50 ml of 10x PBS + 450 ml dH$_2$0, filtrate before use using syringe filters
- Trypsin use to detach tumor epithelial cells when confluent (Thermo Fisher Scientific, Cat. # 12604013)
- Pierce$^{TM}$ 10X Tris-Glycine SDS Buffer (Thermo Fisher Scientific, Cat. # 28362)
- RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Cat. # 89900)

## 3.4   List of equipment

- Automatic pipettes 10-200 μl and 300-1000 μl (Eppendorf)
- Centrifuge 5810R (Eppendorf)
- Cold room
- Electronic pipette controller (Eppendorf)
- Equipment to detect fluorescence: EnSpire Multimode Plate Reader 2300-001M (Perkin Elmer)
- Freezer (Thermo Fisher Scientific)
- Hemocytometer (Sigma-Aldrich, Z359629)
- Incubator (Thermo Fisher Scientific)
- Microscope AX10 (Carl Zeiss)
- Multi-channel 8 pipette (Eppendorf)
- Plates black OptiPlates 384 well (Perkin Elmer)
- Plates white OptiPlates 384 well (Perkin Elmer)
- Flow cytometer (Frotess Becton Dickinson)
- Scientific 2D graphing and statistics software Microsoft Office 365
- Syringe filters 0.2 μm (Sigma-Aldrich) to sterilize CuEt solutions
- Trans-Blot Turbo Transfer System (Bio-Rad)
- Dry Block Thermostat (Biosan)
- Lab Orbital shaker
- Chemiluminiscence detector
- Minicentrifuge
- Electrophoresis chamber and gel casting kit (Bio-rad), prepared 10% polyacrylamide gel, nitrocellulose membrane

## 3.5 Methods

### 3.5.1 Isolation of splenocytes

Splenocytes were obtained from the spleens of mice. Spleens were transferred to Petri dishes with phosphate-buffered saline (PBS) and 1 mM EDTA and were mechanically digested using a scalpel and sterile syringe. Mechanical digestion resulted in rupture of the spleen, disruption of the marrow and release of splenocytes. The suspension of individual cells and smaller aggregates was pipetted through several layers of sterile gauze into a second tube and topped up with PBS. Subsequently, the samples were washed with PBS by centrifugation at 1500 rpm for 10 minutes. The cells were then resuspended in 1 ml of PBS, counted, adjusted to $4.10^5$ and placed in a 37°C humified water jacked incubator with 5% carbon dioxide for use in experiments.

### 3.5.2 Cultivation of tumor cells

Mouse melanoma cell line B16F10 was cultured in culture bottles with DMEM in an incubator at 37 °C. Passaging with a maximum limit of 15 was performed every 2-3 days. Before the counting, the medium containing dead cells was removed from the vials. Trypsin was added to cleave peptide bonds. After trypsin treatment, the adherent cells were scraped from the vial walls and the cell suspension with trypsin was transferred to a tube and topped up with DMEM. Subsequently, the samples were washed with DMEM by centrifugation at 1200 rpm for 10 minutes. After a final wash, cells were resuspended in 1 ml DMEM, counted, and adjusted to $6 \cdot 10^5$.

### 3.5.3 Counting of cells

Cell number was determined by measuring cell viability with trypan blue dye. In a microtube, 10 µl of cell suspension was mixed with an equal volume of trypan blue solution. From the prepared mixture, 10 µl was pipetted into the hemocytometer. The hemocytometer was covered with a coverslip and placed under a 20x10 magnification light microscope objective. Live cells (clean; dead - blue) were counted in randomly selected squares. The number of cells counted was then multiplied by 2, which is the dilution factor (sample : trypan blue - 1:1), $10^4$ to give the result in cells per ml, and then by a number representing the original sample volume (in ml). The cells were then adjusted by the addition of complete medium, splenocytes to 400 thousand cells per ml and tumor cells to 600 thousand cells per ml.

### 3.5.4 Cytotoxicity assays

**Preparation of the cells for the assays**

The cytotoxicity assay was used to evaluate the effects of CuET on cytotoxicity. CuET was first dissolved in DMSO at a concentration of 2.5 mM and then diluted to complete medium to 1 nM. Splenocytes were incubated with the prepared 1 nM DMSO. Tumor cell lines were incubated with 1 nM DMSO for 18 h. After incubation, cells were washed in PBS and used for cytotoxicity text.

For cytotoxic assays, the following combinations were used:

1. Control non treated lymphocytes, and non treated tumor cell lines (15x repeated)
2. Non treated lymphocytes, and 1nM CuET treated tumor cells (18 h) (5x repeated)
3. Treated lymphocytes with 1 nM CuET for different time points (1 h, 2 h, 20 h and 24 h), and non treated tumor cells (15x repeated)

**Cytotoxicity assay**

Effector cells (lymphocytes) were incubated with target cells (B16F10) at different effector-to-target ratios (1:6, 1:5, 1:4, 1:3, 1:2, 3:5, 2:3, 3:4, 4:5, 1:1, 6:5, 4:3, 3:2, 2:1, 3:1, 4:1, 6:1) for different time intervals (1 h, 2 h, 20 h, 24 h) at 37 °C in a 384-well plate. The suspension of treated or untreated lymphocytes was pipetted in duplicates, and a constant amount of splenocytes was pipetted into the plate columns. The smallest volume of lymphocytes pipetted into the each well of first two columns of the plate was 8 µl. In each of the next two columns, the volume of lymphocytes pipetted was increased by 8 µl (8 µl, 16 µl, 24 µl, 32 µl, 40 µl a 48 µl). Tumor cell suspensions (treated/untreated) were pipetted at a constant volume in the rows of the plate. The smallest volume pipetted into the C-row was 5 µl. In each subsequent row, the volume of lymphocytes pipetted was increased by 5 µl (5 µl, 10 µl, 15 µl, 20 µl, 25 µl and 30 µl). DMEM up to 200 µl was added to each well. A and B- line were used for control.

The plates were then incubated in an incubator at 37 °C for various time intervals of 1 h, 2 h, 20 h and 24 h. After incubation, 30 µl of propidium iodide (1 µg/ml in PBS) was added to each well. Subsequently, the plates were incubated for 30 min in the dark before reading the fluorescence of propidium iodide. Propidium iodide, a dye that only penetrates damaged cell membranes, forms complexes with double-stranded DNA. This binding causes fluorescence enhancement.

### 3.5.5 Fluorimetric analysis

After incubation, the plates were centrifuged at 1000 rpm for 1 min to remove possible bubbles in the wells of the plates. A fluorimeter based on propidium iodide fluorescent on dead cells was used for the detection of dead B16F10 cells. Background fluorescence was the fluorescence of tumor cells in the absence of leukocytes. Propidium was excited

with 480 nm radiation, and the results were analyzed at 625 nm. The obtained data were converted to lytic units for easier interpretation of the results and used for statistical analysis.

### 3.5.6 Use of lytic units

The results of cytotoxicity tests are usually given as a percentage of cells killed based on a fixed number of cells. They are calculated according to the equation. Because we used different ratios of effector and target cells in our experiments, percentage analysis would be difficult. For better interpretation of the results, the data were converted to lytic units. A lytic unit can be defined as the number of effector cells required to induce lysis of a certain percentage of target cells. The lower the number of cells required for lysis, the higher the lytic unit and vice versa.

Lytic unit calculation example:

Lytic units in $10^7$ effectors $= (10^7/T \cdot Xp)$

T is the number of target cells (50 000), Xp is the number of cells required to kill 15% of the cells calculated per effector to target ratio as described in the previous equation (2).

$$Lytic\ units = \frac{10^7}{50\ 000 \cdot 2} = 100\ Lytic\ units$$

### 3.5.7 Flow cytometry assay of signal transduction

Flow cytometry analysis was performed using a flow cytometer, Frotress Becton Dickinson, the antibody is labelled with FITC and consequently, the samples were analyzed in the 525 nm filter. For the analysis BD software was used. This experiment was used to evaluate the activation of signal transduction by temporal kinetics with CuET, and the effect of CuET on the kinetics of stimulation. An antibody against phosphorylated tyrosine signaling molecules was used to analyze signal transduction.

Tumor cell lines or splenocytes ($10^5$) were treated at room temperature for 6 different times (1 min, 5 min, 10 min, 15 min, 20 min and 30 min) with four concentrations of CuET (1 nM, 5 nM, 10 nM and 100 nM). After treatment, the samples were cooled to below freezing temperature. Subsequently, 25 μl of permeabilization solution and 10 μl of anti-phosphotyrosine monoclonal antibody conjugated with FITC (diluted 1:10 with PBS) were added to each sample. Subsequently, the samples were incubated for 30 min, washed with PBS, the supernatant was aspirated and the samples were analyzed on a flow cytometer.

### 3.5.8 Western blot

**Sample preparation for Western blotting**

Tumor cells treated with 1 nM CuET, tumor cells treated with 10 nM CuET and positive and negative controls were used for western blot analysis. It was necessary to prepare lysates from tumor cell cultures before western blot analysis. Lysates were prepared by adding lysis buffer (RIPA) with phosphatase inhibitors (1:20) and protease inhibitors (1:50). Afterwards, the samples were vortexed and centrifuged for 30 min at 14000 rpm. The protein concentration of the samples was determined by comparison with standards using the BCA assay in a microtiter plate.

**Electrophoresis**

Sample volumes containing 15-20 μg of protein were mixed 1:4 with leading buffer. The prepared samples were centrifuged on a mini centrifuge, evaporated for 5 min using a Dry Block Thermostat, and after rapid cooling, applied to a 10% polyacrylamide gel. Ladder was applied to the first well of the gel. Subsequently, electrophoresis was performed in 1:10 diluted 10x tris-glycine/SDS buffer, at 80V, after equilibration the levels were V increased 120, and 3A.

**Blotting and antibody labeling**

After electrophoresis, proteins were transferred to the nitrocellulose membrane by electrophoretic transfer in a Trans-Blot Turbo Mini Size. Normalization of Western blotting data, performed to reduce errors caused by uneven sample loading, inconsistent preparation, etc., was performed using Ponceau S dye. Subsequently, blots were blocked in 5% BSA for 2 hours at room temperature with gentle shaking. They were then incubated with anti pPhosphotyrosine (clone PY20) primary antibody against phosphotyrosine (diluted 1:1000 in TBST – a mixture of tris-buffered saline (TBS) and Polysorbate 20) overnight. Afterwards, blots were wash four times with TBST and incubated wuth anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (diluted 1:5000 in milk) for 1 hour. Blots were transferred to a slide with parafilm and treated with the ECL™ Western Blotting System (substrate). After 5 minutes the blots were dried with pulp and measured. The reaction was revealed with an enhanced chemoluminiscence detection method.

### 3.5.9 Statistical analysis

The statistical analysis of the results was performed in the GraphPad Prism 5 program (GraphPad Software, Inc.) and Excell using one-way analysis of variance (ANOVA), followed by Bonferroni t test for multiple comparisons. BD software was used to analyze the flow cytometry results.

# 4 RESULTS

## 4.1 Cytotoxicity assay – CuET increased lymphocyte cytotoxicity

Modulation of immune reactions (in this case cytotoxic reactions) is highly relevant in tumor cell destruction. To assess CuET-induced cytotoxicity, the B16F10 cell line and mouse splenocytes were un/treated with CuET, and cytotoxicity was measured as described in Chapter 3.5.4 and 3.5.5. The data obtained were converted to lytic units as described in Chapter 3.5.6 and plotted in Graph 1. The results showed that CuET treatment of any cells increased the cytotoxicity of C57BL/6 lymphocytes against the F16B10 cell line.

Pre-treatment of lymphocytes with 1 nM CuET induced a significant increase in lytic activity against untreated B16F10 target cells at all treatment time intervals (1 h, 2 h, 20 h and 24 h). The most pronounced increase in cytotoxicity compared to control (point 0) was measured for treated lymphocytes – untreated tumor cell combination after 20 h of treatment (Graph 1. For this combination, cytotoxicity decreased after 24 h of treatment. The effect of CuET on lymphocyte cytotoxicity was time dependent. The combination of untreated lymphocytes against B16F10 tumor cells treated with 1 nM CuET did not result in such a significant increase in lytic activity (approximately 1.5-fold lower than the opposite combination) (Graph 1). This suggests that the cytotoxic effect was more pronounced when immune cells were treated with CuET as opposed to treated tumor cells.
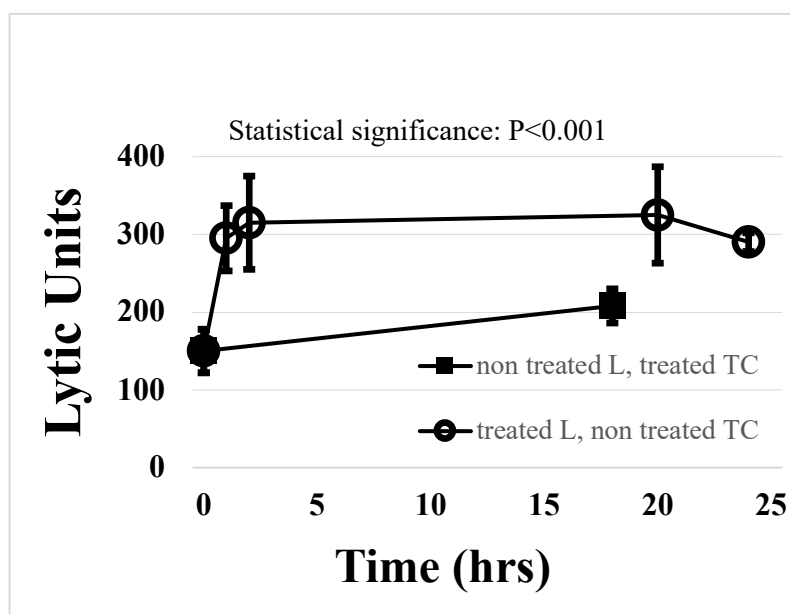


*Graph 1: Cytotoxic activity of T cells against the B16F10 cell line. Point 0 - control - untreated tumor cells + untreated splenocytes. o – 1 nM CuET treated tumor cells + untreated lymphocytes, ◢ – untreated tumor cells + 1 nM CuET treated lymphocytes. Statistical significance: P<0.001.*

## 4.2 Analysis of tyrosine phosphorylation after CuET treatment by flow cytometry

Tyrosine phosphorylation controls many cellular functions including cell signalling, growth and division, and gene expression. T cell activation also leads to tyrosine phosphorylation of several molecules (including TCR chain molecules). Protein tyrosine phosphorylation was assessed by flow cytometry as described in Chapter 3.5.7. Kinetic experiments were performed to determine the stimulation time that elicited the best phosphotyrosine signal intensity for both lymphocytes and tumor cells.

CuET-treated lymphocytes did not show significant stimulation of tyrosine phosphorylation and the peaks of measured fluorescence varied for different concentrations of CuET (Graphs 2A-2D). Lymphocytes stimulated with 1 nM CuET showed the highest tyrosine phosphorylation after 20 min of stimulation, 5 nM after 10 min of stimulation, and 10 nM and 100 nM after 30 min of stimulation.

The responses of B16F10 tumor cells to CuET treatment increased during the first 5 minutes of stimulation at concentrations of 1 nM, 5 nM, and 10 nM as seen in Graphs 2A-2C. The maximum phosphotyrosine fluorescence signal was repeatedly measured after five minutes of treatment at these CuET concentrations, and then gradually decreased. In B16F10 cells treated with 100 nM CuET, the fluorescence showed a small statistically insignificant increase after 1 min of treatment. For cells treated with 100 nM CuET for more than 5 min, the measured fluorescence was lower than that of the control (point 0) (Graph 2D). From the data obtained, the treated cells showed a time-dependent increase in tyrosine phosphorylation to one peak and then a gradual decrease. The effect of the treatment dose of CuET on signal amplitude was also examined. The graph shows that increasing CuET concentration results in an increase in phosphorylated tyrosine levels in tumor cells, at least up to a treatment concentration of 5 nM (Graph 2B). In tumor cells treated with 10 nM CuET or more, phosphorylation is lower than in cells treated with 1 nM. In lymphocytes, the treatment concentration of CuET has no significant effect on tyrosine phosphorylation. The graphs 2A-2D shows data from five experiments.
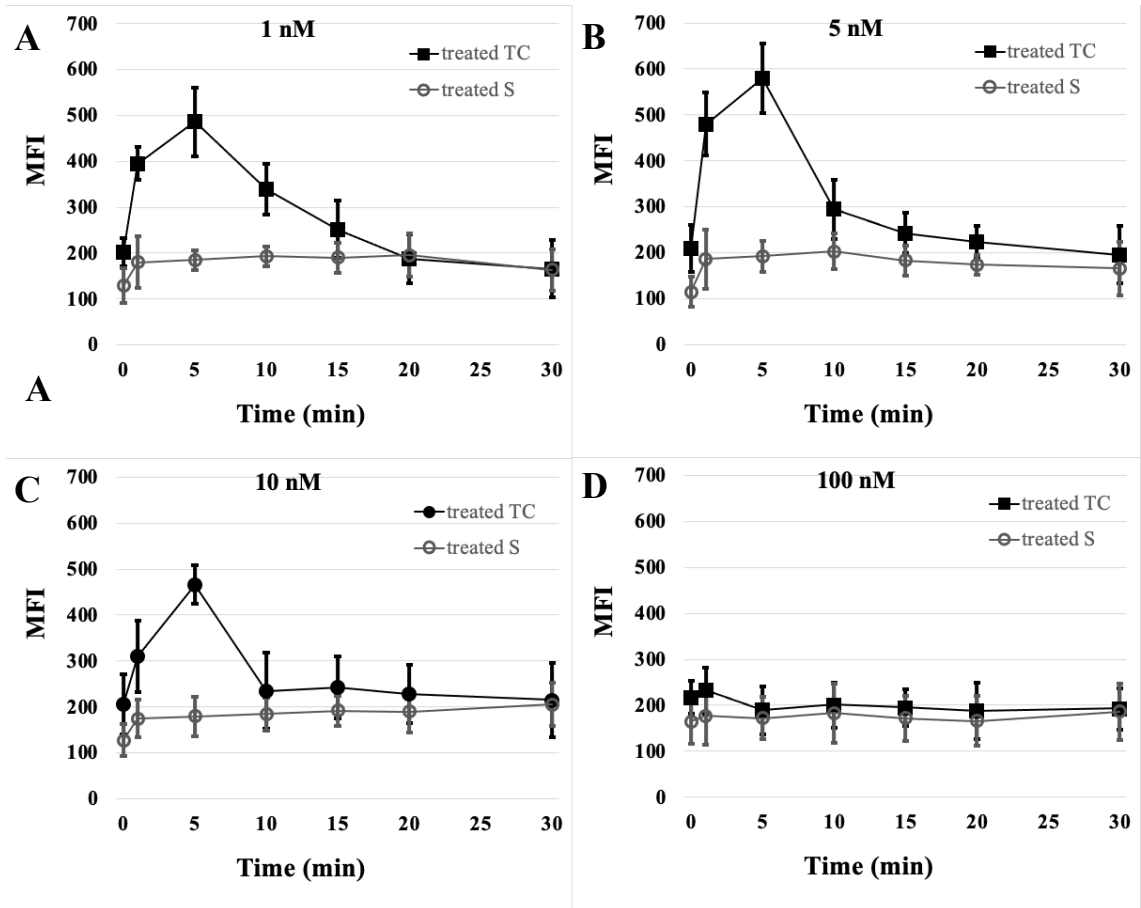
Graphs 2A-2D: Assessment of signal transduction activation by time kinetics with CuEt. Kinetic of stimulation with CuEt 1 nM (A), 5 nM (B), 10 nm (C) a 100 nM (D). Statistical significance for treated splenocytes: P<0.05, 1 nM – 5, 10, 15, 20 min, 5 nM – 5, 30 min, 10 nM – 20 min; P<0.001 1 nM – 5, 10 min, 5 nM – 10, 15, 20 min, 10 nM – 1, 5, 10, 15 min. Statistical signifikcance for treated tumor cells: P<0.01, 1 nM 1 min, 10 nM 1 min, P<0.001, 1 nM – 5, 10 min, 5 nM – 1, 5 min, 10 nM – 5 min.

## 4.3 Western blot

Immunoprecipitants from CuET-treated tumor cells and lymphocytes were also tested for the presence of phosphorylated tyrosine by Western blotting, according to the procedure described in Chapter 3.5.8. Western blot analysis using the anti-phosphotyrosine antibody PY20 confirmed that treatment of both lymphocytes and tumour cells with 1 nM and 10 nM CuET increased the levels of tyrosine-phosphorylated proteins (Figure 9). Figure 10 show one of five identical experiments.

The pY20 antibody stains a larger number of proteins across the entire ladder range. For both types of cell lysates, several proteins with phosphorylated tyrosine's are visible on the gel. Phosphorylation increased in lymphocytes in response to CuET stimulation predominantly for proteins of around 40 kDa in size, and further for proteins of 55 kDa and 100-110 kDa in size. In lysates from tumor cells, phosphorylation also increased predominantly for proteins of around size 40 kDa and further for 50, 55 and 100 kDa. There was no apparent difference between the western blot results of samples treated with different concentrations of CuET (1 nM, 10 nM).
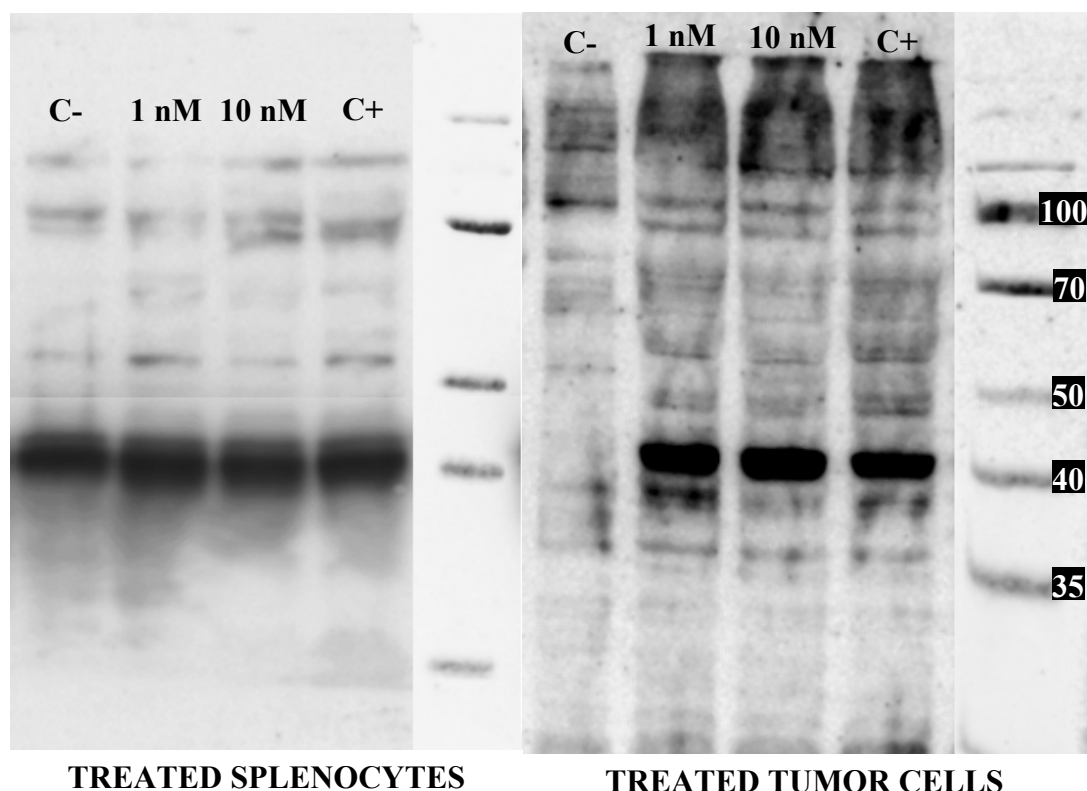


**TREATED SPLENOCYTES**          **TREATED TUMOR CELLS**

*Figure 10: Detection of the effect of 1 nM and 10 nM CuET on tyrosine phosphorylation by Western blotting with anti-phosphotyrosine antibody PY20*

# 5 DISCUSSION

Research and development of anticancer drugs is often plagued by long treatment cycles and high costs. To save medical resources, the anticancer effects of traditional, originally non-cancer drugs are currently being investigated.

Disulfiram (DSF) is a thiuram derivative that has been used for more than 70 years to treat alcohol dependence. However, DSF has also been found to have anti-cancer activity. Copper (Cu), as a trace metal, has numerous roles in the body. DSF is metabolized in vivo and reacts with trace copper to form copper diethyldithiocarbamate (CuET), for which the responsibility of DSF for anticancer activity has been confirmed in several preclinical and clinical studies (Huang et al., 2021). However, the mechanisms of action of CuET have not yet been fully elucidated. Initial studies investigated the antitumor effect of DSF by inhibiting the proteasome or acetaldehyde dehydrogenase, but both of these theories have been refuted (Skrott et al., 2017). A promising possible mechanism for the antitumor activity of DSF has been proposed by the Skrott et al., 2017 – DSF targets tumors via the p97 segregase adaptor NPL4 involved in the catabolism of folded proteins. However, this discovery is very recent and only further studies can show whether this mechanism will be confirmed or refuted.

In the context of mechanisms of action, the effect of CuET on the induction of cytotoxic response was investigated in this study. There was a significant increase in cytotoxicity against untreated target cells after CuET treatment. The cytotoxicity was probably induced by increased production of cytotoxic receptors in effector cells by affecting signaling cascades. The cytotoxicity of CuET-untreated lymphocytes against treated tumor cells was probably based on the induction of antigen expression by tumor cells, but the lytic activity was much lower. This effect may also have been due to downregulation or inhibition of the PD1/PD-L1 checkpoint. PD-1/PD-L1 blockade leads to attenuation of the inhibitory signal, restoration of the efficacy of immune mechanisms, and increased antitumor activity, which may be related to increased phosphorylation. Thus, treatment with 1 nM CuET was confirmed to increase lymphocyte cytotoxicity against B16F10 cell lines, leading to increased cell death and B16F10 cells becoming more accessible to immune re after treatment. After more than 20 hours of treatment, the cytotoxic effect began to decrease.

Regarding the evaluation of signal transduction activation by flow cytometry kinetics in CuET-treated cells. CuET-treated lymphocytes showed no significant difference in the

stimulation of tyrosine phosphorylation. In CuET-treated tumor cells, there was a significant time-dependent increase in tyrosine phosphorylation up to one peak (5 min) and then a gradual decrease. The maximum phosphotyrosine fluorescence signal was repeatedly measured after five treatments with 5 nM CuET. However, there is almost no increase in phosphorylated tyrosine's in samples treated with more than 100 nM CuET. The results show that there was a significant increase in phosphorylation in response to CuET treatment, predominantly in tumor cells.

To visualize the size of the proteins involved in tyrosine phosphorylation, we performed Western blotting, and from the results we found the presence of proteins with phosphorylated tyrosines of about 40, 50, 55, 90-100 and 130 kDa in both types of treated cells. The most intense phosphorylation was observed for proteins around 40 kDa in size; such large proteins could correspond to tyrosine phosphorylated ERK1/2. ERK1/2 signaling controls T cell development, differentiation, and the strength of TCR-induced signaling (Shah, Al-Haidari, and Sun). Proteins of 90-100 kDa could correspond to tyrosine-phosphorylated STAT proteins, around 130 kDa JAK proteins and others. Further analyses would be required to pinpoint specific proteins. In general, however, there was tyrosine phosphorylation of several proteins of different sizes after CuET treatment in both types of cell lysates, which is evidence for the progression of cell signaling. We suggest that the cell signaling-based phosphorylation caused by CuET treatment results in the induction of antigen expression in tumor cells and the stimulation of lymphocytes to react against antigens expressed by the tumor.

# 6 CONCLUSION

This work focused on the effect of CuET on cytotoxic response and signal transduction by tyrosine phosphorylation of proteins. The output data showed that CuET treatment enhanced lymphocyte cytotoxicity against the murine melanoma cell line B16F10. This was through stimulation of lymphocytes, which is likely to affect the production of cytotoxic receptors in effector cells. By flow cytometry, it was found that there is a significant increase in tyrosine phosphorylation in tumor cells after CuET treatment, indicating the activity of one of the signaling pathways. The activated signaling pathway may result in the expression of proteins, in the case of tumor antigens, that are subsequently recognized by immune cells.

In summary, our results suggest that CuET has a significant effect on both the cytotoxic response and tyrosine protein phosphorylation, probably by two mechanisms: activation of signaling pathways for antigen expression by tumor cells, and increased production of cytotoxic receptors by lymphocytes. Although DSF has made progress in cancer treatment, research is still ongoing. While the exact mechanisms of action of CuET have not yet been fully elucidated, the efficacy of DSF itself is well known and its safety profile has been explored, making repurposing DSF and the resulting CuET a promising strategy as a novel anticancer drug. We can conclude that CuET is a potentially promising anticancer drug.

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# 8    LIST OF ABBREVIATIONS

APC                    Antigen presenting cell

BCR                    B cell receptor

CD4                    Helper T cells

CD8                    Cytotoxic T cells

CuET                   Diethyldithiocarbamate-copper complex

DNA                    Deoxyribonucleic acid

DSF                    Disulfiram

DSH                    Diethyldithiocarbamate

ERK                    Extracellular signal-regulated kinase

IgG                    Imunoglobulin G

JAK                    Janus kinase

LAK                    Lymphokine-activated killer cell

MAPK                   Mitogen-activated protein kinase

MHC                    Major histocompatibility compley

NFkB                   Nuclear factor kappaB

NK                     Natural killer cell

PTP                    Protein tyrosine phosphatase

pTyr                   Phosphotyrosine

ROS                    Reactive oxygen species

TAA                    Tumor associated antigen

TCR                    T cell receptor

TSA                    Tumor specific antigen

TyrK                   Tyrosine kinases

UV                     Ultraviolet