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Antigen presentation and immune response in cancer: Effect of Disulfiram metabolites

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SUMMARY

The present thesis deals with the antigen presentation and immune response in cancer, namely the effect of Disulfiram metabolites.

In the theoretical part, cancer is briefly described and in particular chronic myeloid leukemia along with the cell lines used in the experiments. Antigen presentation and immune response in cancer followed by immunotherapy are discussed next. In the last chapters, we focused on disulfiram and its metabolites and their use not only in cancer treatment.

The experimental part focuses on the enhancement of cytotoxic response which can be due to an increase in NK cell activity or antigen presentation, increasing specific T lymphocyte cytotoxicity. It was found that a disulfiram metabolite treatment enhances NK and T cell cytotoxicity against the K562 and K562 TAX cell lines. One of the proposed mechanisms is the activation of NF-κB. Inhibition of NF-κB should abolish the effect of disulfiram metabolites on NK and T cytotoxic responses. The NF-κB Inhibitors: N acetylcysteine and aspirin together with sulfamethoxazole as a control were used for this experiment. We showed that the inhibition of NF-κB by N acetylcysteine and aspirin abolished the effect of disulfiram metabolites on NK and T cytotoxic responses against the K562 and K562 TAX cell lines. These results confirm that activation of NF-κB could be one of the mechanisms responsible for disulfiram effect against cancer.

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SOUHRN

Tato bakalářská práce se zabývá tématem prezentace antigenu a imunitní odpověď při rakovině, konkrétně účinkem metabolitů Disulfiramu.

V teoretické části je stručně popsána rakovina, a to zejména chronická myeloidní leukémie spolu s buněčnými liniemi použitými v experimentech. Jako další je probrána prezentace antigenu a odpověď při rakovině, následovaná imunoterapií. V posledních kapitolách jsme se zaměřili na disulfiram a jeho metabolity a jejich využití nejen při léčbě rakoviny.

Experimentální část pojednává o zvýšení cytotoxické odpovědi, která může být způsobena zvýšením aktivity NK buněk nebo prezentací antigenu zvyšující specifickou cytotoxicitu T lymfocytů. Bylo zjištěno, že ošetření metabolitem disulfiramu zvyšuje cytotoxicitu NK a T buněk proti buněčným liniím K562 a K562 TAX. Jedním z navrhovaných mechanismů je aktivace NF-κB. Inhibice NF-κB by měla zrušit účinek metabolitů disulfiramu při NK a T cytotoxické odpovědi. Pro tento experiment byly použity NF-κB inhibitory: N acetylcystein a aspirin spolu se sulfamethoxazolem jako kontrolou. Dokázali jsme, že inhibice NF-κB pomocí N acetylcysteinu a aspirinu vedla ke zrušení účinku metabolitů disulfiramu na NK a T cytotoxické odpovědi proti buněčným liniím K562 a K562 TAX. Tyto výsledky potvrzují, že aktivace NF-κB by mohla být jedním z mechanismů zodpovědných za účinek disulfiramu proti rakovině.

DECLARATION

I hereby declare that I wrote this bachelor thesis independently under the supervision of De Sanctis Juan Bautista, prof. Ph.D., using only information sources stated in the references.

In Olomouc, …… ……..

Eva Komínková

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I would like to express my gratitude to my supervisor De Sanctis Juan Bautista, prof. Ph.D., for his guidance and patience during the work on my thesis. I am very grateful to Janošťáková Anna M.Sc., for her training in the Tissue Culture Laboratory and for sharing her knowledge. I want to acknowledge the financial support from Molecular and Cellular Clinical Approach to Healthy Ageing (ENOCH project to MH, JdS) via IMTM (#869/V19) and Grants of the Ministry of School Education and Youth of the Czech Republic (CZ.02.1.01./0.0/0.0/16_019/0000868 and LM2015064)

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LIST OF ABREVIATIONS

APC	Antigen presenting cells
CAR	Chimeric antigen receptor
CML	Chronic myelogenous leukemia
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte–associated antigen 4
CuET	Diethyldithiocarbamate copper
DC	Dendritic cell
DDTC	Sodium diethyldithiocarbamate/Imuthiol
DMSO	Dimethyl sulfoxide
DSF	Disulfiram
DTC	Diethyldithiocarbamate (a metabolite of DSF)
DβH	Dopamine β hydroxylase
FBS	Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
TAAs	tumor-associated antigens
TCR	T-cell receptor
Treg	T regulatory cell

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1 INTRODUCTION

In recent years, epidemiological data analysis on different drugs revealed that disulfiram an Antabuse drug could be used in cancer. Since disulfiram and its metabolite diethyldithiocarbamate have been discovered in the 1940s, many pharmacological assays have tried to ascertain the mechanism of action of both drugs. Disulfiram, but not its metabolite diethyldithiocarbamate, inhibits acetaldehyde dehydrogenase, the key enzyme responsible for the detoxification of alcohol. Nonetheless, both compounds were shown to affect several signalling pathways. Treatment with disulfiram or diethyldithiocarbamate *ex vivo* or *in vivo,* in the mouse model, was shown to potentiate immune response, decrease autoimmune disease and activate cytotoxic response against tumours. However, no precise mechanism has been published of how both compounds are able to enhance the immune response.

Our aim was to study the enhancement of cytotoxic response in cancer which can be due to an increase in NK cell activity or antigen presentation, increasing specific T lymphocyte cytotoxicity. Moreover, we will focus on a plausible mechanism, the activation of NF-κB. The increase in both cytotoxic responses induced by DSF and its metabolite copper complex CuET could be due to increased production of the cytokine interferon-gamma. NF-κB is a protein complex that controls cytokine production; therefore, its inhibition should abolish the effect of disulfiram metabolites on NK and T cytotoxic responses.

2 AIMS OF THE THESIS

- Gather literary resources and elaborate the theoretical review on the topic of the bachelor thesis.
- Ascertain NK and T cytotoxic response against K562 and K562TAX cells in the presence of disulfiram metabolites. Cell death will be assessed by propidium iodide emission using a fluorimeter.
- Study whether the inhibition of NF-κB will abolish the effect of disulfiram metabolites on NK and T cytotoxic responses.

3 LITERATURE REVIEW

3.1 Cancer

A hundred years ago, cancer incidence was low; however, since the last couple of decades, its rate has been rising alarmingly. This phenomenon might be caused by our lifestyle changes, habits, and increased average human life expectancy. The probability nowadays is that one of four people have a lifetime risk of cancer. (Roy *et* Saikia, 2016).

What is usually called 'cancer' includes more than 200 different diseases related to abnormal cell proliferation. Cancer cells typically contain multiple aberrations in the number and structure of chromosomes as well as in genes. The majority of genetic alternations found in cancer cells are gained by mutations in somatic cells; only a few are caused by genetic defects obtained during fetal development (Schulz, 2007).

Despite the increasing knowledge gained on the molecular mechanisms involved in the deregulation of cancer cells, such as oncogenes and oncosuppressor genes identification, numerous questions about the origin of cancer cells have not been answered yet (La Porta *et* Zapperi, 2017).

3.1.1 The origin of cancer

Cancer cells do not respond appropriately to control mechanisms and signals that affect the cell cycle. They do not show contact inhibition and continue to divide uncontrollably, although growth factors are consumed. Such unusual behaviour of cancer cells in the body can have fatal consequences. The problem begins when one cell undergoes a malignant transformation-the process of converting a conventional cell into a cancer cell. The transformed cell is usually recognized by the body's immune system as an intruder and destroyed. However, if the cell escapes immune surveillance, it can multiply and give the basis of tumour-accumulation of abnormal cells within normal tissue (Campbell *et* Reece, 2006)

If abnormal cells remain only in their original position, the tumour is referred to as benign. Most benign tumours do not cause severe problems and can be removed entirely by surgery. In contrast, malignant tumours invade surrounding tissues and can metastasize-spread using the circulatory system to other parts of the body. These metastases disrupt healthy tissue and organ function and are responsible for 90% of cancer mortalities. (Campbell *et* Reece, 2006; Jensen *et al.,* 2011).

Normal cell genes, called proto-oncogenes, encode proteins that stimulate healthy cell growth and division. Cancer-causing genes are called oncogenes. The products of proto-oncogenes and oncosuppressor genes direct cell division. A change in DNA can make a proto-oncogene excessively active and transform it into an oncogene that can promote excess cell growth and cancer. The

oncosuppressor gene encodes a protein that suppresses abnormal cell division. As an example, can be given a key oncosuppressor gene which is of fundamental importance for cancer development: P53, also known as the "guardian of the genome." Loss or mutation of such a gene has a similar effect to oncogene activation. Tumor cells accumulate changes affecting proto-oncogenes and oncosuppressor genes. Some of these mutations may be inherited, making them predisposed to the development of cancer (Campbell *et* Reece, 2006).

3.1.2 Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cell origin arising when bone marrow stem cells produce excessive and abnormal white blood cells (Clarke *et* Holyoake, 2017; Hewison *et al.*, 2020). It is a rare disease more common in males than females with European incidence of 1–2 cases per 100 000 adults increasing by age but all age groups, children included, can be affected. In Europe, the median age at diagnosis of CML is 57–60 years, which is about 10 years above the median age typically seen in clinical trials (Höglund *et al.*, 2015; Hewison *et al.*, 2020).

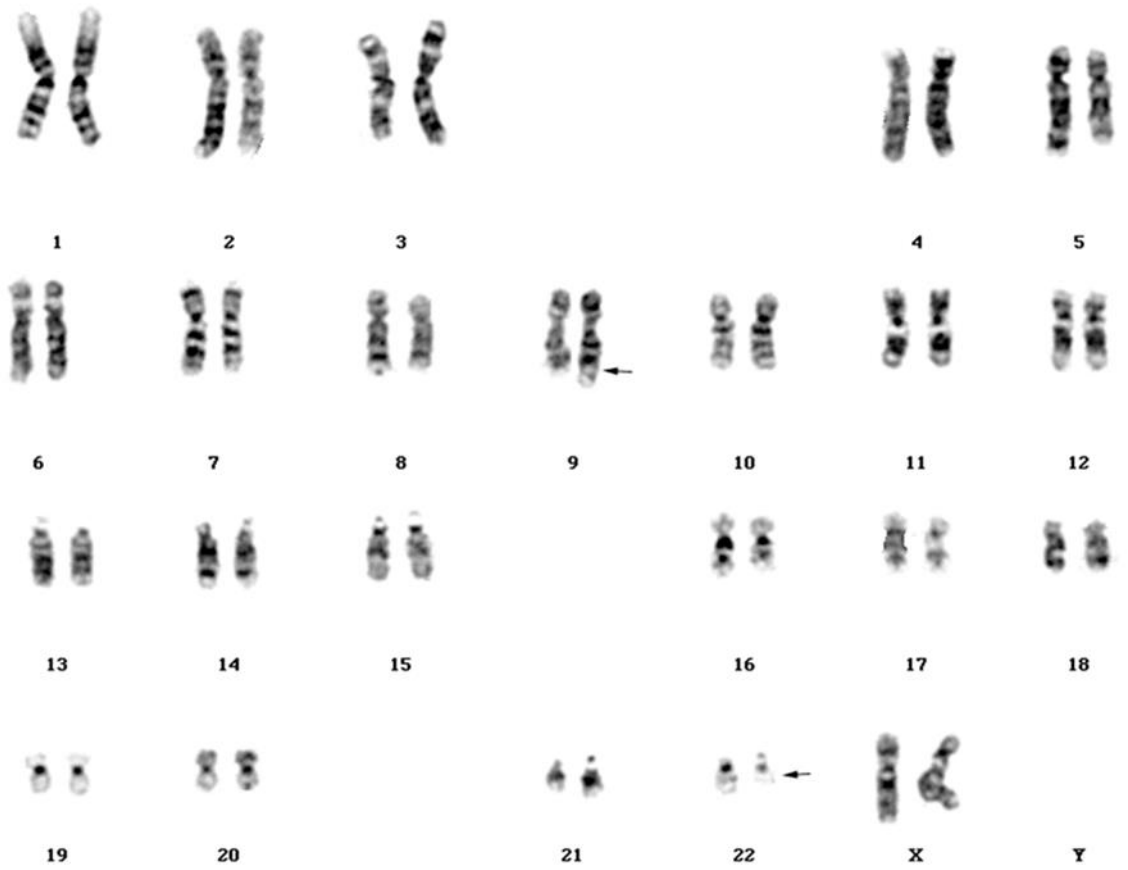


Figure 1: Human karyotype – chronic myeloid leukemia (the Philadelphia chromosome), t (9;22). (From: https://wellcomecollection.org/works/t4dsq2x7) [visited March 21st, 2020].

CML karyotype is characterized by the presence of the Philadelphia (Ph) chromosome (Figure 1), which is found in up to 95 percent of patients. The abnormality comes from a reciprocal translocation, t (9;22) (q34; q11.2) between the long arms of chromosomes 9 and 22, involving a fusion of the Abelson gene (ABL1) from chromosome 9q34 with the breakpoint cluster region (BCR) gene on chromosome 22q11.2. (Faderl *et al*., 1999; Jabbour *et* Kantarjian, 2018). As a consequence, the generation of a BCR-ABL1 fusion oncogene translates into a BCR-ABL1 oncoprotein. This constitutively active tyrosine kinase can influence significant signalling pathways involved in cell survival, proliferation, adhesion, and differentiation. (Clarke *et* Holyoake, 2017).

The majority of patients are diagnosed in the chronic phase of the disease, which, if untreated, progresses to an accelerated phase and ultimately to fatal blast crisis within 3 to 5 years (Figure 2). The typical symptoms at presentation are commonly associated with anemia and splenomegaly such as fatigue, weight loss, malaise or easy satiety Around 40 percent of patients are asymptomatic, and disease diagnosis is based on an abnormal blood count (Sawyers, 1999; Jabbour *et* Kantarjian, 2018).

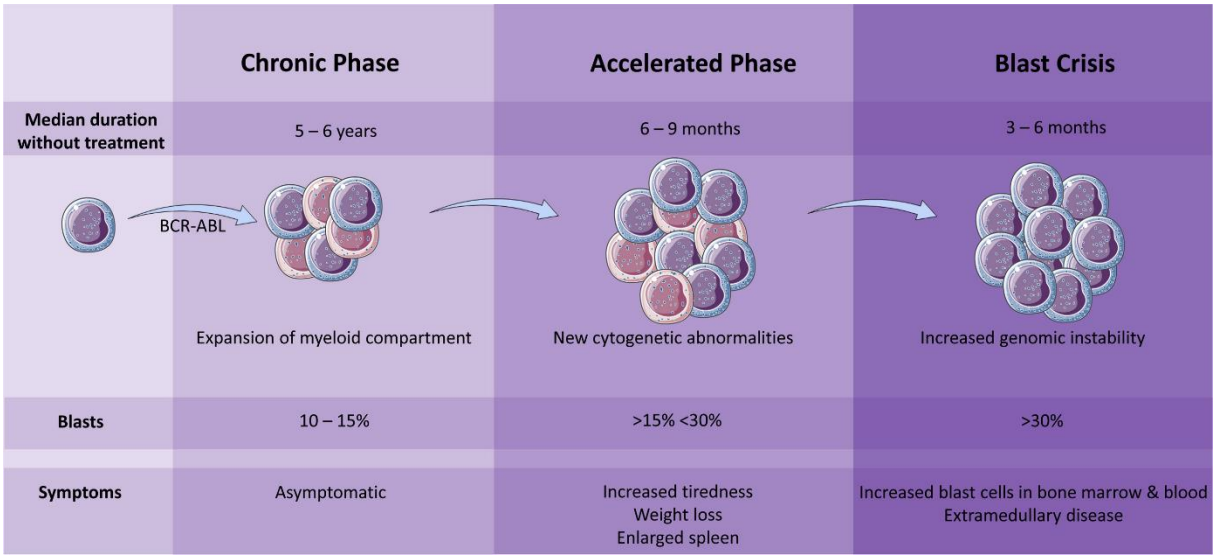


Figure 2: CML disease progression (From: Clarke *et* Holyoake, 2017).

Each phase can be characterized by the number of immature cells (blasts) found in the bone marrow. Expression of BCR-ABL1 oncoprotein activates several signalling pathways, resulting in increased proliferation and decreased apoptosis in the myeloid compartment. Secondary genetic and molecular abnormalities lead to an accumulation of mutations and genomic instability, resulting in progression to blast crisis and poor patient prognosis (Clarke *et* Holyoake, 2017).

3.1.2.1 K562 and K562-TAX cell line

A non-adherent K562 is a cell line established *in vitro* from a pleural effusion of a 53-year-old female with chronic myeloid leukaemia in terminal blast crisis (Lozzio *et* Lozzio, 1973). K562 was selected for the experiments for several reasons - the main reason is that this cell line is suitable for assessing spontaneous cytotoxicity, natural killer activity. Also, it can be used to generate antigen-dependent, T cell cytotoxicity. The cell line is also very easily maintained as a stationary suspension culture (Klein *et al*., 1976).

K562-TAX, also known as KPTA5, is an adherent paclitaxel-resistant cell line established by co-selecting the parental erythroleukemic cell line K562 with stepwise increased concentrations of paclitaxel (Taxol®) in the presence of the cyclosporin D analogue PSC 833. K562-TAX cells are 9- fold resistant to paclitaxel (Jaffrézou *et al.,* 1995). K562 TAX is resistant to NK spontaneous cytotoxicity (Geromin *et al*., 2004).

3.2 Antigen presentation and immune response in cancer

Antigen presentation is essential for inducing an immune response; the problem is that tumor antigen expression is highly heterogeneous, and the process of antigen presentation involves many obstacles in the tumor microenvironment. Before antigen can be recognized and generate an effective adaptive response, it needs to be processed by the organelles of the cell so a specific antigenic peptide, or epitope, is later externalized. There exist two types of molecules capable of presenting the processed antigens to the cells responsible for the adaptive immunity, major histocompatibility complex (MHC) MHC class I and MHC class II molecules. Whereas, MHC class I molecules present peptides to CD8+ cytotoxic T cells and are expressed by all nucleated cells, MHC class II molecules present peptides to CD4+ helper T cells and only professional antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells, are able to express them. Mature APCs are crucial for initiating an effective adaptive immune response (Cezero *et al*., 2015; Cezero-Wallis *et* Soengas, 2016).

The immune system needs to distinguish between healthy cells and malignant tumour cells to fight cancer. Unfortunately, it is not as simple as it might seem because tumours use many mechanisms to avoid immune recognition. These mechanisms include creating highly immunosuppressive tumor microenvironment by increased production of cytokines, down-regulating or neutralizing self-antigens, the expression of MHC class II molecules on tumor cells is decreased. Thus, T cytotoxic cells are not able to recognize them as well as tumor

mutations cause the loss of antigens capable of induction of immune response (Šťastný *et* Říhová, 2015; Cezero-Wallis et Soengas, 2016).

Many cells of the innate and adaptive immunity play an essential role in the antigen presentation and immune response in cancer, including macrophages, mast cells, neutrophils, B lymphocytes and others. However, we will focus on dendritic cells, T cells and NK cells.

3.2.1 Natural Killer cells (NK cells)

NK cells belong to the innate lymphoid cells, although it also exhibits features of adaptive immunity. NK cells kill spontaneously tumours that lack MHC class I molecule expression, or they kill tumour cells coated with specific antibodies by antibody-dependent cell-mediated cytotoxicity. Cytokines control the development and functional maturation of NK cells. People with defects in NK cell function or numbers caused by genetic mutations are more prone to develop tumours than the general population, indicating that NK cells are important in antitumor-activity (Cezero-Wallis et Soengas, 2016; Abbas *et al.*, 2017; Wu *et al.*, 2017; Gaynoe *et* Colucci, 2017).

3.2.2 Dendritic cells

Dendritic cells (DCs) are one of the pillars of the innate immunity. They are considered to be the most potent antigen presenting cells since they belong to professional APCs which are able to present antigens through MHC class I and also MHC class II (Cezero-Wallis *et* Soengas, 2016). DCs scan the peripheral tissue for antigens, and after their recognition antigens are internalized, DCs are activated and migrate toward the draining lymph node, where they can induce an adaptive immune response (Figure 3). Therefore, these cells control cytotoxic and antibody-dependent responses (Embgenbroich *et* Burgdorf, 2018).

3.2.3 T cells

The killing of tumour cells by $CD8^+$ Cytotoxic T lymphocytes (CTLs) is the primary specific mechanism of immune protection against cancer. Dendritic cells have the ability to capture tumor cells and present the tumor antigens to naïve CD8+ CTLs. Tumor antigens are picked up by DCs and carried to lymph nodes where are colocalized with naïve T cells. Then, naïve $CD8^+$ T cells differentiate into tumour-specific CTLs with the help of DCs and helper $CD4^+$ T cells. Finally, tumour-specific CTLs migrate back to the tumour and kill it, see figure 3 (Abbas *et al*., 2017).

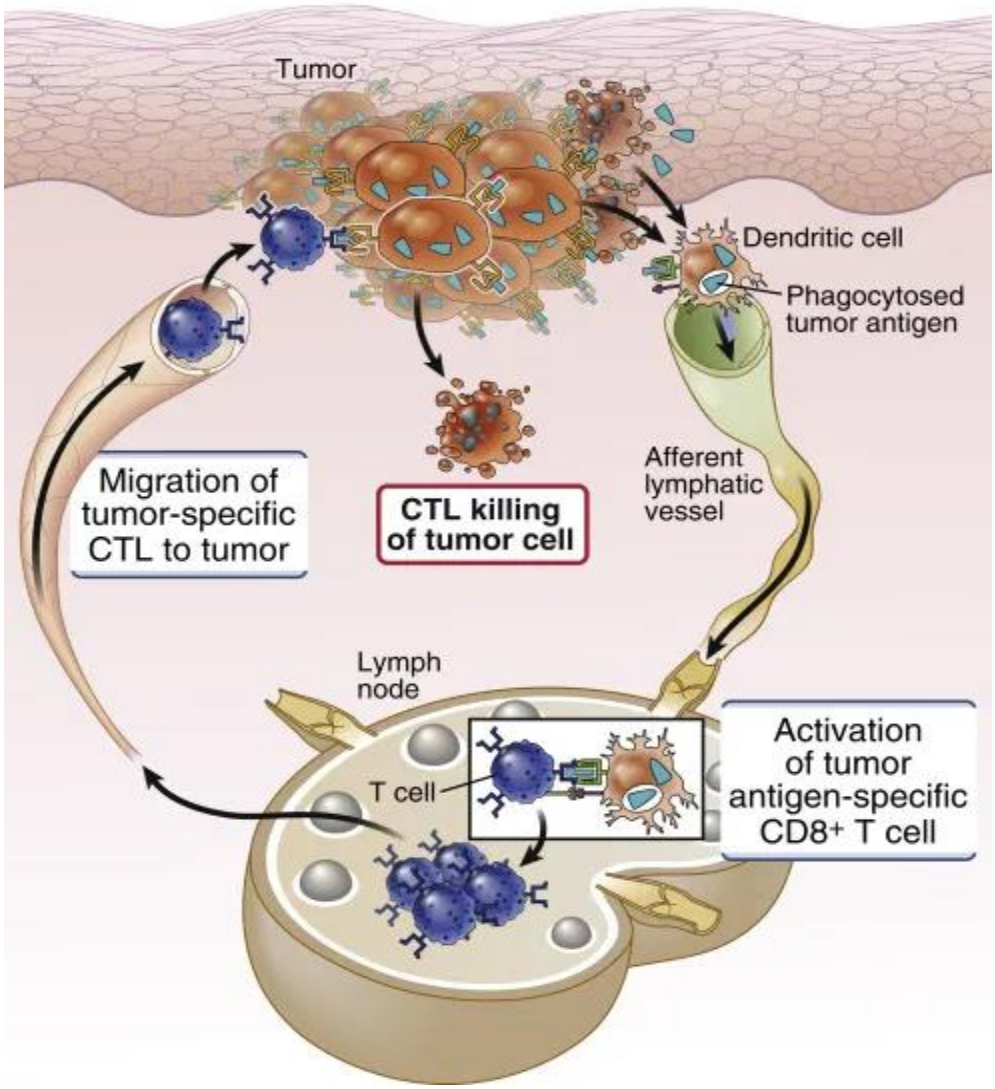


Figure 3: Cytotoxic T lymphocyte (CTL) response against tumors. (From: Abbas *et al.*, 2017)

3.3 Tumor immunotherapy

The beginnings of cancer immunotherapy date back to 1863, when Rudolph Virchow described immune infiltrates (Figure 4). Nowadays, immunotherapy is revolutionizing, which caused that over 2000 immuno-oncology agents are currently under scrutiny. Cancer immunotherapy was chosen by Science's editors as 2013's Breakthrough of the Year. In 2018, James P. Allison and Tasuku Honjo received the Nobel Prize in Physiology or Medicine for their development of cancer therapy by the blockade of co-inhibitory signals (Couzin-Frankel, 2013; Bakacs *et al*., 2019).

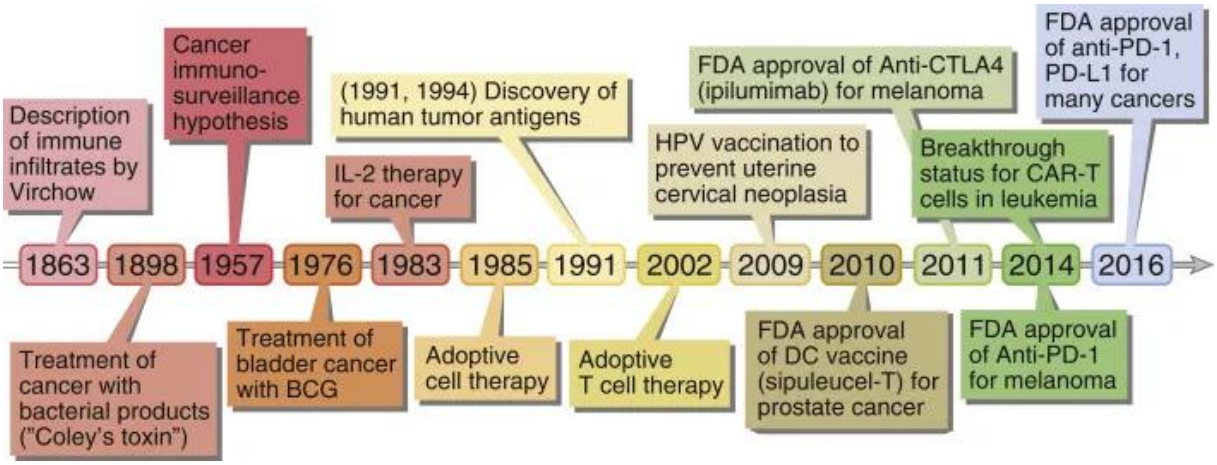


Figure 4: History of cancer immunotherapy (From: Abbas *et al.*, 2017)

There exist many approaches regarding the cancer immunotherapy, e.g. the usage of peptide vaccines for cancer therapy (Cezero *et. al*, 2015) or cytokine therapy when patients are treated with cytokines that stimulate the proliferation and differentiation of T lymphocytes and NK cells (Abbas *et al.*, 2017) and many others. Nevertheless, two approaches stand out and mark the beginning of a new era in cancer immunotherapy; these include immune checkpoint blockade therapy using antibodies that block CTLA-4 and PD-1 T cell inhibitory pathways and chimeric antigen receptor (CAR) T cell therapy (Yang, 2015).

CTLA-4 and PD-1 are inhibitory receptors that are expressed on activated T cells once the resolution of inflammation is generated. They bind to ligands either on APC (CTLA-4 binding to CD80/CD86) or tumour cells (PD-1 binding to PD-L1). Since they are inhibitory receptors, they recruit phosphatases that suppress cell activation and consequently, the antitumor response. CTLA-4 is the inhibitory receptor of the CD28 family expressed on the surface of activated $CD4^+$ and $CD8^+$ T lymphocytes for B7 molecules. CTLA-4 is responsible for inhibition of the early stages of T lymphocyte activation. Antibodies that target CTLA-4 are

slowing tumour progression in many patients suffering from several malignancies, such as melanoma and renal cell carcinoma. These antibodies probably work not only by blocking the action of CTLA-4 on effector T cells but also on Tregs.

Another inhibitory receptor of the CD28 family is PD-1 that recognizes two ligands PD-L1 and PD-L2. PD-1 is expressed on antigen-activated T cells and assists in T cell deactivation in peripheral tissues. Checkpoint blockade with anti-PD1 and anti-PD-L1 turns on T cell-mediated immunity which has a positive effect on many malignancies. The FDA approved antibody inhibitor for CTLA-4 is Ipilimumab, and for PD-1 the antibody inhibitors are Pembrolizumab and Nivolumab. A combined immune checkpoint blockade of both CTLA-4 and PD-1 is more effective than monotherapy against certain cancers, including melanoma and renal-cell cancer. (Abbas *et al*., 2017; Alsaab *et al*., 2017; Szostak *et al.,* 2019). The general principle of cancer checkpoint blockade therapy can be seen in figure 5.

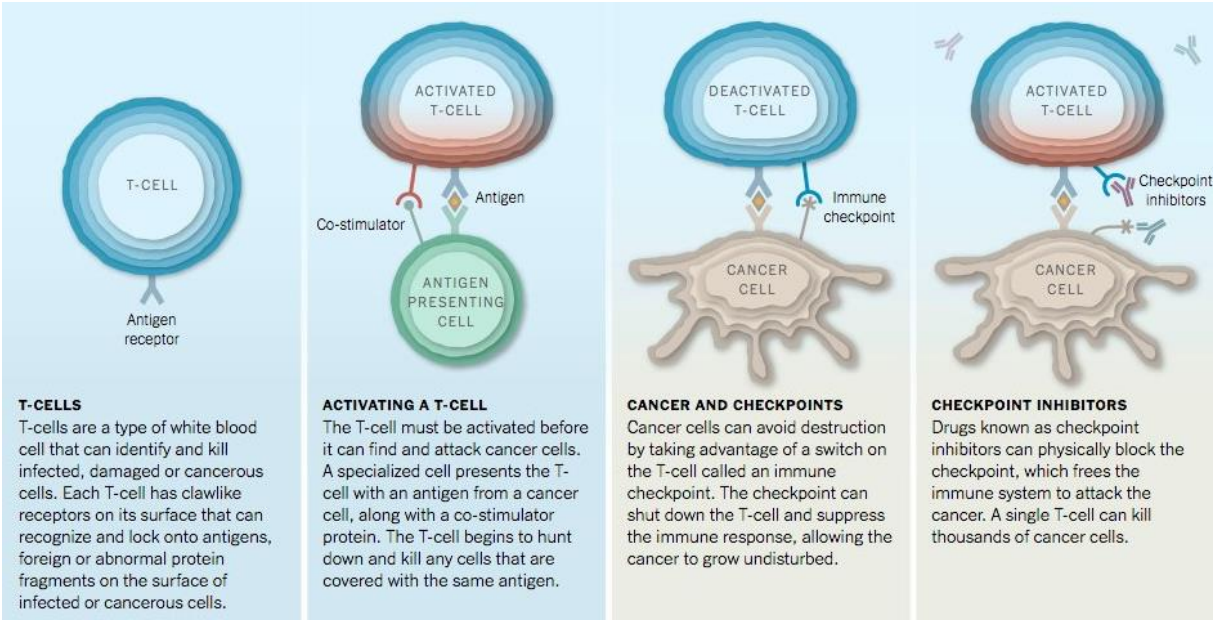


Figure 5: How immunotherapy works. (From: https://www.nytimes.com/2018/10/01/health/nobel-prize-medicine.html) [visited June 18th, 2020].

CAR T cells are autologous T lymphocytes genetically engineered to express the antigen-binding region of an antibody directed against tumour-associated antigens (TAAs). CAR T cells consist of three parts: single-chain variable domain of an antibody, a transmembrane domain, and a signal transduction domain of the T-cell receptor (TCR). CAR T cells mediate MHC-unrestricted tumour cell killing by enabling T cells to bind target cell surface antigens through a single-chain variable fragment recognition domain. The therapy consists in the isolation of T cells from the blood of a patient, their stimulation with anti-CD3 or anti-CD28 antibodies,

followed by genetic modification to express recombinant CARs, after which they are transferred back into a patient undergoing robust proliferation. When the CAR engages with a specific antigen, T cell activation occurs via the signal transduction domain of the TCR. Tumour killing is achieved by direct cytotoxic as well as cytokine-mediated mechanisms. So far, CAR T cell therapy was fruitful only against hematologic malignancies because the injected T cells can easily access the circulating tumour cells. In case of solid tumours, T cells must be injected into the tissue site of the tumour which is not doable at this time (Abbas *et al*., 2017; Benmebarek *et al.*, 2019; 8., Heyman *et* Yang, 2019).

3.4 Disulfiram and its metabolites not only in cancer treatment

3.4.1 Disulfiram in the treatment of alcohol dependence

Disulfiram (Figure 6), better known as Antabuse, is a Food and Drug Administration (FDA) approved drug for the treatment of alcohol dependence and has been available for clinical use for over six decades (Kona *et al.*, 2011). Disulfiram (tetraethyl thiuram disulfide) is a derivative of thiuram and was initially used as an industrial catalyst for rubber production in the 1800s. In 1937, the American chemical plant physician E. E. Williams observed that chemical plant workers regularly exposed to Disulfiram (DSF) experienced unpleasant physiological symptoms after alcohol intake. These findings made him think about DSF as a potential cure for alcoholism (Jiao *et al*., 2016).

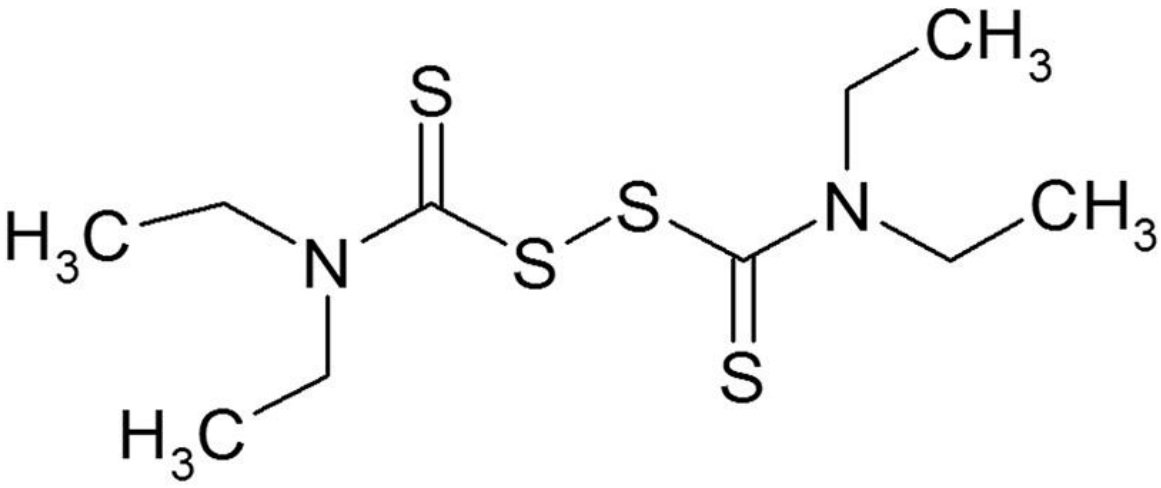


Figure 6: Structure of Disulfiram (Bis(diethylthiocarbamoyl) disulfide) (From Jiao *et al.*, 2016).

The rationale for disulfiram to treat alcoholism is that it inhibits the enzyme aldehyde dehydrogenase (ALDH), that is responsible for converting acetaldehyde to acetate in metabolizing alcohol. Disulfiram inhibits ALDH which increases the concentration of acetaldehyde. The

accumulation of acetaldehyde results in unpleasant reactions which occur immediately after drinking alcohol. These reactions include facial flushing, sweating, mild headache escalating in heart palpitations and breathing difficulties. The extent of the unpleasant effect that each person experiences depends on the amount of alcohol and disulfiram dosage (Suh et al., 2006).

Williams observations were published in 1937 (Williams, 1937). The report was ignored until 1945, when Hald and Jacobsen ingested DSF for the experimental treatment of intestinal worms and inadvertently took prandial wine afterwards, resulting in the unpleasant reactions mentioned above. Thus, by serendipity, DSF started to lure attention to the treatment of alcohol dependence (Hughes et Cook, 1997). In 1948, some clinical trials confirmed the anti-alcoholic properties of DSF, and the FDA approved its application for the treatment of alcoholism (Jiao et al., 2016).

3.4.2 Anticancer activity of disulfiram and its metabolites

Cancer incidence is rising, and this global challenge is deepened further by tumor resistance to available therapies. Furthermore, in most countries, healthcare service budgets are not able to support the research of new oncology drugs. A promising approach to this situation is repurposing the large arsenal of approved, non-anticancer drugs such as DSF because of cheaper, faster and safer preclinical and clinical validation protocols. In recent years, epidemiological data analysis on different drugs revealed that DSF, an old alcohol-aversion drug, could be used in cancer as cancer patients treated with disulfiram survived longer than their untreated counterparts (Bertolini *et al.*, 2015; Skrott *et al.*, 2017).

Dr Lewison published the very first article about the clinical anticancer activity of DSF from Johns Hopkins Hospital in Baltimore in 1977. This article deals with the case of a 35-year-old woman who suffered from breast cancer with numerous bone metastases (spine, skull, pelvis and ribs). Since she became a severe alcoholic, all hormone therapy, chemotherapy and radiation therapy had to be discontinued and replaced with a DSF treatment. Over the next 10 years of disulfiram treatment metastases have completely disappeared and the patient remained clinically free of cancer without any form of further anticancer therapy until her death when she accidentally fell from a third-floor window due to drunkenness (Lewison, 1977).

In the 1990s, a group of French scientists initiated a phase II clinical trial of adjuvant immunotherapy with sodium ditiocarb (DDC), metabolite of disulfiram, versus placebo in high risk breast cancer. Sixty-four patients operated on with non-metastatic high-risk breast cancer were randomized into two equal groups for immunotherapy. Group 1 received standard chemotherapy along with DDC, whereas group 2 received standard chemotherapy but with placebo. The treatment started with the first chemotherapy cycle and lasted nine months. After

five years, six metastatic relapses were observed and five deaths in DDC group as compared to 13 metastatic relapses and 12 deaths in the placebo group. The overall survival was significantly higher in DDC group (81% vs 55%). This study revealed that DDC associated with chemotherapy and locoregional treatment could improve survival in high risk breast cancer (Dufour *et al.*, 1993). Unfortunately, the authors did not realize that sodium dithiocarb is the main metabolite of disulfiram. Thus, the trial has been forgotten even though it was successful (Cvek, 2012).

Encouraging results were also achieved in 2015 in a phase II trial assessing the addition of DSF to chemotherapy for the treatment of metastatic non-small cell lung cancer. Twenty patients were treated with and twenty without DSF. The results were that patients in the disulfiram group lived on average of three months longer than the ones in the control group. The unusual result was the long-term survival of two patients in the DSF group, an event that is extremely rare in patients with stage IV lung cancer treated by chemotherapy alone, whereas patients in the control group all died within two years (Nechusthan, 2015).

The potential anticancer activity of DSF was also confirmed in subsequent studies. In a 2004 epidemiological study, DSF was shown to have a protective effect against breast and prostate cancer (Askgaard *et al.*, 2014) or a case report where DSF in combination with zinc gluconate induced clinical remission in a patient with stage IV ocular melanoma metastatic to the liver (Brar *et al.*, 2004). DSF is also very efficient *in vitro* in carcinomas of the ovary, breast and in non-small cell lung cancer (Wickström *et al.*, 2007). Matsuno and co-workers suggested that diethyldithiocarbamate could be a promising therapy for primary effusion lymphoma (Matsuno *et al*., 2012).

It was found that copper at intracellular concentrations can bind with DSF and the metal–drug complex has been shown to possess higher levels of antitumor efficacy than DSF (Cen *et al.*, 2004). Following studies indicate that the cytotoxicity of DSF is copper (Cu) dependent. The growth-inhibitory and apoptosis-inducing effects of the DSF-copper complex in breast cancer cells were determined (Chen *et al*., 2006). Moreover, disulfiram was found to be highly toxic to breast cancer cell lines *in vitro* in a copper (Cu)-dependent manner and inhibited breast cancer stem cells (Yip *et al*., 2011).

3.4.2.1 Possible mechanisms of the anticancer activity

So far, a lot of misleading information regarding possible mechanisms of DSF anticancer activity has been published. Therefore, in the following lines, I will try to clarify these ambiguities and gathered information regarding possible DSF anticancer mechanisms.

After oral ingestion, if copper is available DSF is in the acidic environment of the stomach rapidly converted to its complex with copper known as CuET (Figure 7). Afterwards, CuET is uniformly distributed throughout bloodstream into the whole body (Johansson, 1992). As already mentioned above, DSF has been shown to possess higher levels of antitumor efficacy in the presence of copper, indicating that the cytotoxicity of DSF is copper dependent (Cen *et al*., 2004; Chen *et al*., 2006).

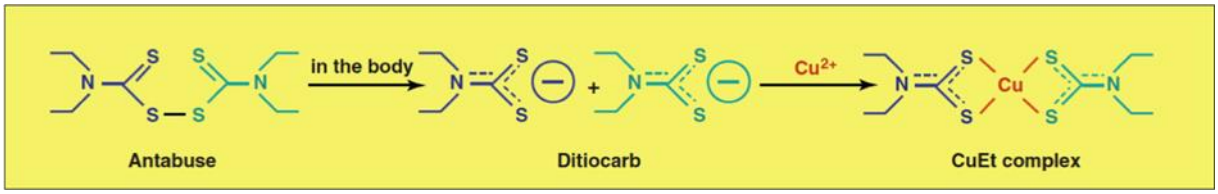


Figure 7: Antabuse is rapidly metabolized to ditiocarb (diethyldithiocarbamate) in the human body. Ditiocarb as a strong cooper chelator reacts with copper forming Cu(II) diethyldithiocarbamate complex (CuET) (From: Cvek, 2012).

In 2006 CuET was shown to be a potent proteasome inhibitor and apoptosis inducer in human breast cancer cells but not in normal, immortalized human breast cells. Thus, the anticancer activity of DSF started to be associated with its proteasome-inhibitory and apoptosis-inducing abilities. Incidentally, the ability to induce apoptosis in the tumour, but not healthy, cells is an essential criterion for novel anticancer drugs (Chen *et al.*, 2006).

Nuclear factor-kappa B (NF-κB) is a transcription factor, which plays a vital role in cell proliferation and malignant transformation. In one study, Disulfiram-mediated inhibition of NF-κB activity led to increased cytotoxicity of 5-fluorouracil in human colorectal cancer cell lines (Wang *et al.*, 2003). In another study, the NF-κB activity in Glioblastoma multiforme cells was significantly inhibited by CuET. Although NF-κB is an attractive molecular target for therapeutic intervention, inhibition of NF-κB alone only induces limited cell death (Liu *et al*., 2012) indicating that several factors are involved in the antitumor activity of CuET. In the practical part of the thesis was used a different approach regarding NF-κB, see section 5.

Recently, Skrott *et al*. identified CuET as the ultimate anti-cancer metabolite of DSF responsible for its anticancer effects. Moreover, they also revealed the long-sought molecular

target of disulfiram's tumour suppressing effects as NPL4, an adapter of p97/VCP segregase essential for protein turnover involved in multiple regulatory and stress-response cellular pathways, indicating that DSF targets cancer via p97 segregase adapter NPL4. By this discovery, they refuted that proteasome is the DSF target as predicted in previous studies (e.g Chen *et al.*, 2006) since neither 20S nor 26S proteasome were involved. However, the processing of ubiquitylated proteins by the NPL4-dependent segregase is targeted by CuET. Their proposed model of DSF anticancer activity in patients can be seen in figure 8 (Skrott *et al.*, 2017).

Two years later, Skrott *et al.* refuted another generally accepted theory that the antitumor activity of DSF involves ALDH inhibition. They conclude that the antitumor activity of DSF rather reflects the impact of CuET, which kills cells through aggregation of NPL4, a subunit of the p97/VCP segregase (Skrott, *et al.*, 2019).

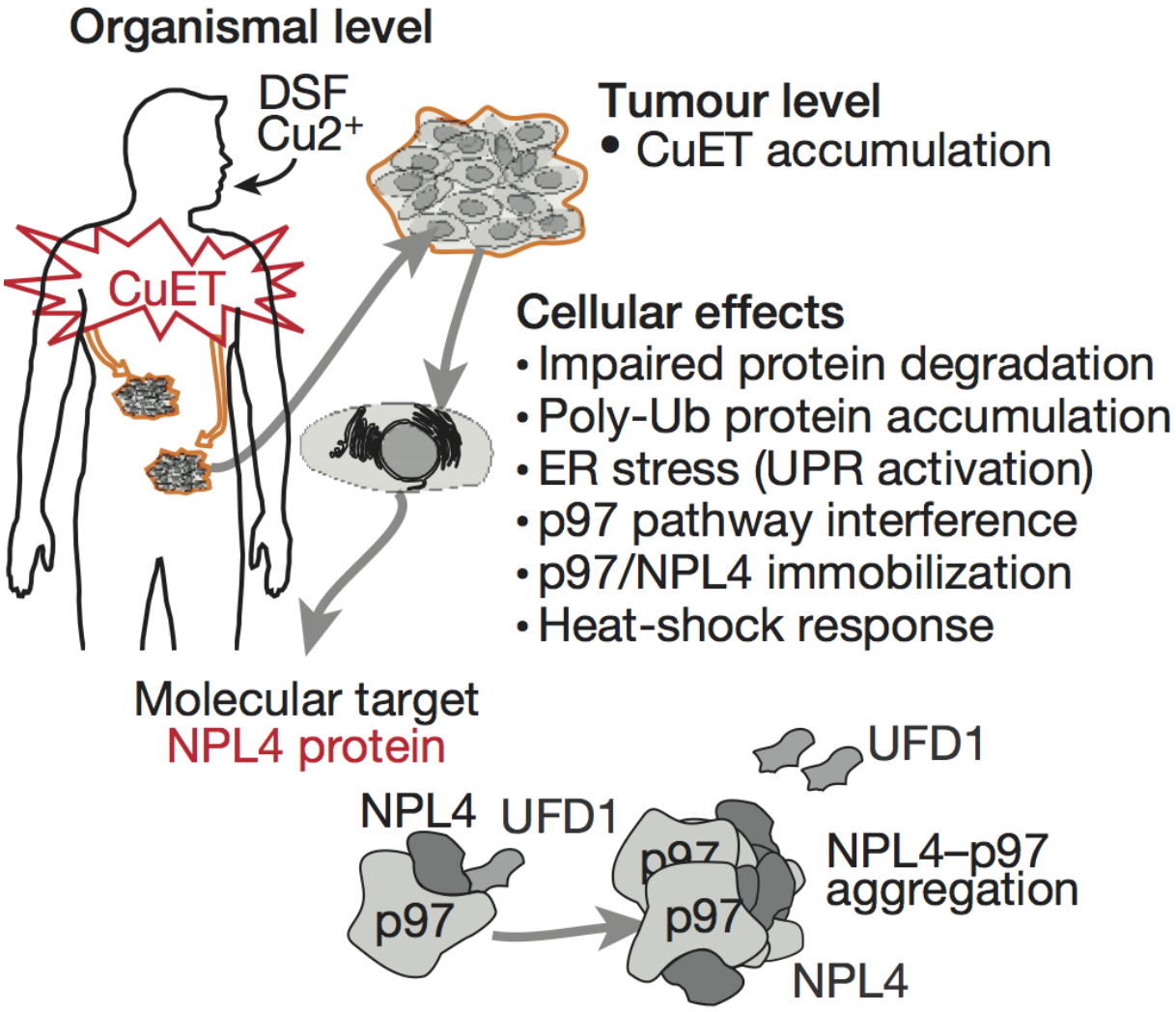


Figure 8: Model of DSF anti-cancer activity in patients (From: Skrott *et al.*, 2017).

3.4.3 Other uses of disulfiram and its metabolites

It was found that disulfiram and its metabolites are beneficial not only in alcohol dependence and cancer treatment. Because the association between alcohol and cocaine dependence is very common, researchers have recently begun to focus on the possibility of using disulfiram in the treatment of cocaine dependence. Recent clinical trials showed that DSF effectively decreases cocaine consumption in cocaine-dependent patients and alcohol + cocaine-dependent patients.

However, the mechanism of action of DSF is dissimilar from the one regarding alcohol, and it is not entirely understood (Suh *et al.*, 2006; Gaval-Cruz *et* Weinshenker, 2009). One potential explanation is that disulfiram inhibits the oxidoreductase dopamine β hydroxylase (DβH), an enzyme which converts dopamine to norepinephrine. The inhibition of DβH results in very high levels of dopamine. Therefore, the usage of cocaine during disulfiram treatment leads to psychosis, paranoia and increased anxiety. All these unpleasant consequences seem to deter cocaine use (Suh *et al.*, 2006; Kampangkaew *et al.*, 2019).

In late 1980s, there was a report that sodium diethyldithiocarbamate (DDTC), Imuthiol, reduced the symptoms and improved immunology parameters in patients with AIDS-related complex (ARC) in clinical trials caused a big upheaval (Lang *et al.*, 1985; Lang *et al*., 1988). These findings were soon confirmed by Hersh et al., 1991. However, more extensive trials completely refuted previous positive results with the conclusion that Imuthiol had no beneficial effect on the progression of the HIV disease and the manufacturer withdrew Imuthiol from the clinical investigation (Vanham *et al.*,1993; THE HIV87 STUDY GROUP 1, 1993).

Nevertheless, a few years later was suggested that such contradictory results might be due to the fact that the drug in the body can react with several dietary components, e.g. metal salts, so that the reaction products may hamper the effect of the drug; some researchers are trying to solve the enigma (Cvek, 2009). After that, new trials regarding the use of Disulfiram in HIV therapy started arising. The results revealed that disulfiram activates HIV transcription through depletion of the phosphatase and tensin homolog (PTEN). This inhibition results in the activation of the Akt signalling pathway initiating proviral transcription in an NF-κB-dependent manner. (Rasmussen *et* Søgaard, 2018; Spivak *et* Planelles, 2018). Several chemical compounds, along with DSF, have been identified as HIV as latency reversing agents (LRAs) and clinical trials are being prepared to ascertain these effects. Although more clinical trials and studies are still needed, DSF selectivity for myeloid cells suggests that DSF could represent a good LRA candidate to reactivate neglected reservoir of myeloid origin including monocytes and microglial cells. (Kula *et al.*, 2019)

According to Halpern *et. al* the metabolite of DSF Diethyldithiocarbamate (DTC) has been shown to increase survival in mice with spontaneously developing systemic lupus erythematosus-like disease. Their findings suggest that DTC might be a useful treatment for this autoimmune disease (Halpern *et al*., 1990; Halpern *et* Yocum, 1991).

4 MATERIAL AND METHODS

4.1 Biological material

The following cell lines were used for the experiments, K562 purchased from ATCC and taxol resistant K562 TAX/KPTA5 provided by Dr Jean Pierre Jaffrezou.

The cells were grown in RPMI medium enriched by 10% Fetal Bovine Serum and antibiotics streptomycin (100 $U \cdot ml^{-1}$) and penicillin (100 $U \cdot ml^{-1}$). The incubation conditions of the cells were at 37 °C in humidified 5% carbon dioxide atmosphere, in a regular water jacked incubator (Thermo Scientific). Both cell lines were passaged every 2-3 days to maintain exponential growth, around 50 000 cells/ml. The passage limit was 15.

Blood specimens were obtained from healthy human volunteer donors, provided by the institution FN Olomouc-Haematological department.

4.2 Chemicals and reagents

- 10% Fetal Bovine Serum (FBS) (Gibco, Cat. # 10270-106)
- Aspirin (Sigma-Aldrich, Cat. # A2093)
- Copper chloride (Sigma-Aldrich, Cat. # 203149)
- Diethyldithiocarbamate (Sigma-Aldrich, Cat. # D3506)
- DMSO (Sigma-Aldrich, Cat. # D4540)
- Ethanol (Sigma-Aldrich, Cat. # 493511)
- Hystopaque 1077 Ficoll-Paque density gradient media (Sigma-Aldrich**,** Cat. # 10771)
- N acetylcysteine (Sigma-Aldrich, Cat. # A9165)
- Penicillin-Streptomycin Mixture (Lonza, Cat. # DE17-602E)
- Propidium iodide (Sigma-Aldrich, Cat. # 81845)
- RPMI 1640 Medium with L-Glutamine (Lonza, Cat. # 12-702Q)
- Sulfamethoxazole (Sigma-Aldrich, Cat. # S7507)
- Trypan blue (Sigma-Aldrich, Cat. # 93595)
- TrypLE (Gibco, Cat. # 12604054)

4.3 List of solutions

- 1x PBS: 10x PBS 80 g NaCl, 2 g KCl, 14,4 g $Na_2HPO_4 \cdot 2H_2O$, 2 g KH_2PO_4, dissolved in 800 ml dH_20 and added to 1 l; preparation: mix 50 ml of 10x PBS + 450 ml dH_20, filtrate before use using syringe filters
- Enriched RPMI medium (500 ml): Add 50 ml of 10% FBS and 50 ml of the antibiotic mix (streptomycin and penicillin, 100 $U \cdot ml^{-1}$) to 500 ml of RPMI medium, mix and filtrate before use using syringe filters
- CuET prepared from diethyldithiocarbamate and copper chloride by Dr. Petr Dzubak. Dissolved in dimethyl sulfoxide (DMSO) to final concentration of 2.6 mM

4.4 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)
- Hood Herape Ks p2 flow (Thermo Fisher Scientific)
- Incubator (Thermo Fisher Scientific)
- Inverted microscope AX10 (Carl Zeiss)
- Laboratory water bath WNB (Memmert)
- Multi-channel 8 pipette (Eppendorf)
- Plates black OptiPlates 384 well (Perkin Elmer)
- Scientific 2D graphing and statistics software GraphPad Prism 5 (GraphPad Software, Inc.)
- Syringe filters 0.2 µm (Sigma-Aldrich) to sterilize CuET solutions

4.5 Methods

4.5.1 Isolation of mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from human blood healthy donors were purified by standard Ficoll-hypaque density gradient centrifugation. The Ficoll-hypaque solution, 5 ml, was pipetted in 15 ml tubes and then two volumes of donor's blood (10 ml) were carefully added on the top. The blood samples were centrifuged for 30 minutes at 2 000 rpm. The PBMC were harvested from the 'buffy' layer and washed three times with PBS by centrifugation at 1 800 rpm for 10 minutes. Afterwards, cells were resuspended in 1 ml of RPMI 1640 medium, counted and adjusted at $2 \cdot 10^6$/ml. The cells were transferred to a 75 cm^2 cultivation flasks in complete media in order to separate the adherent monocytes from lymphocytes. Then, the cells were incubated in a 37 °C humidified water jacked incubator with 5% carbon dioxide for a minimum of 1 hour. Each experimental point refers to a different donor.

4.5.2 Counting of the cells

Cell viability was determined by trypan blue exclusion. 90 µl of cells in the medium were mixed with 10 µl of trypan blue. From the prepared mixture, 10 µl were pipetted into a hemocytometer, covered with a coverslip and placed under an inverted microscope. Cells were counted in randomly selected squares bounded by two lines. The sum of counted cells was then multiplied by $10^{5,}$ which represents the number of cells per ml. The cells were then adjusted to 2 million/ml by adding the complete media.

4.5.3 Cytotoxicity assays

Preparation of the cells for the assays

PBMCs were incubated in 6-well plates for 24 hours with 1 nM and 10 nM CuET in complete media. Tumor cell lines were incubated for 24 hours with 1 nM CuET in complete media. After the incubation, the cells were washed with PBS and used for the cytotoxicity assays.

For cytotoxic assays, the following combinations were used:

1) control non treated lymphocytes and non-treated tumors
2) treated lymphocytes with 1 or 10 nM CuET for 18 hours, and non-treated tumor cells
3) non treated lymphocytes and 1nM CuET treated tumor cells
4) both effector and target cells treated with CuET 18 hours prior the assay.

10 nM treatment was discarded in the T cell cytotoxicity assays as in NK cell cytotoxicity assays the higher concentrations of CuET were used the lower the cytotoxicity was shown, for confirmation of this hypothesis 50 nM CuET was used and the cytotoxicity was truly lower than when 10nM CuET was used for the treatment

NK cytotoxicity assay (spontaneous cytotoxicity)

Effector cells (PBMCs) were incubated with target cells (K562/K562 TAX) at different effector-to-target (E:T) ratios (20:1, 10:1, 5:1 and 2:1) in sextuplicate for each donor in 384-well plates for 4 hours at 37° C. 50 000 target tumour cells were used constantly in 100 μl of culture media per well. After the 4 hours incubation, 20 µl of propidium iodide (1mg/ml in PBS) was added to each well. The plates were incubated in the dark for two hours before reading the propidium iodide fluorescence (section 4.5.5).

K562 cells are the standard cell lines for NK spontaneous cytotoxic response, K562 Taxol resistant cell lines are not killed efficiently by NK cells.

T cell activation in culture (antigen dependent cytotoxicity)

PBMCs were incubated in culture media with tumor cells fixed with Ethanol at ratio 1:1 for 7 days at 37° C in order to generate cytotoxic T cells against tumor cells and verify the specificity of antigens. After seven days, effector cells (PBMCs) were washed and either treated with 1 nM CuEt 18 hours or not treated and rechallenged with live target cells (K562/K562 TAX) at different effector-to-target ratios as described for NK cytotoxicity assay.

4.5.4 Effect of NF-kB inhibitors

The NF-κB Inhibitors: N acetylcysteine and aspirin were used for this experiment. Sulfamethoxazole was used as a control. PBMCs from 10 different blood donors were stimulated with 1nM CuET for 18 hours, as described previously, washed with PBS to get rid of excess CuET and preincubated in tubes with the inhibitors at increasing concentrations: 1 µM, 10 µM, 20 µM for 30 minutes at 37° C. After the preincubation, the cells were rewashed with PBS and then the cytotoxic assays were performed as described previously.

4.5.5 Fluorimetric analysis

After the incubation, the 384 plates were centrifuged at 1000 rpm for 1 min to disrupt the possible bubbles in the plates. The K562 and K562 TAX cell death was measured using a fluorimeter on the basis of propidium iodide fluorescence on dead cells. Background fluorescence was the fluorescence of leukocytes in the absence of tumor cells, and tumor cells in the absence of effectors, CuET at nM concentrations do not induce cell death. Propidium iodide excites at 480 nm, and the results were analyzed at 625 nm. The obtained results were converted to lytic units and used for statistical analysis.

4.5.6 Use of lytic units

The usual analysis of cytotoxicity is in % killed cells based on the fixed number of tumor cells (50 000) following the equation.

% cytotoxicity:

Background (unspecific fluorescence) 100 fluorescence intensity

Maximum killing/50000 cells 2500 fluorescence intensity

$$\text{Specific cytotoxicity} = \frac{(\text{Value} - 100)}{2500} \cdot 100$$

However, since for each treatment, we have four effectors to target ratios, the analysis of the treatment is difficult to interpret. We used lytic units to normalize the values for each treatment. The lytic units are defined by the inverse of the number of effector cells required to lyse 15 % of the tumour cells. Thus, the lower the number of cells required the higher lytic unit and vice-versa.

Lytic unit calculation:

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

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

For example

Lytic units = 10^7/ 50 000 x 2 = 100 Lytic units

4.5.7 Statistical analysis

The statistical analysis of the results was performed in the GraphPad Prism 5 program (GraphPad Software, Inc.) using one-way analysis of variance (ANOVA), followed by Bonferroni t test for multiple comparisons.

5 RESULTS

5.1. Cytotoxicity assays

Modulation of cytotoxic response is very important in cancer biology. Spontaneous cytotoxicity, NK cells, and antigen-specific cytotoxicity, CD8 cells, are involved in effective antitumor response. In order to assess this effect, PBMCs from 10 different donors were purified by the standard Ficoll- hypaque gradient, treated with CuET and the cytotoxic assays were performed as described in Chapter 4.5.3.

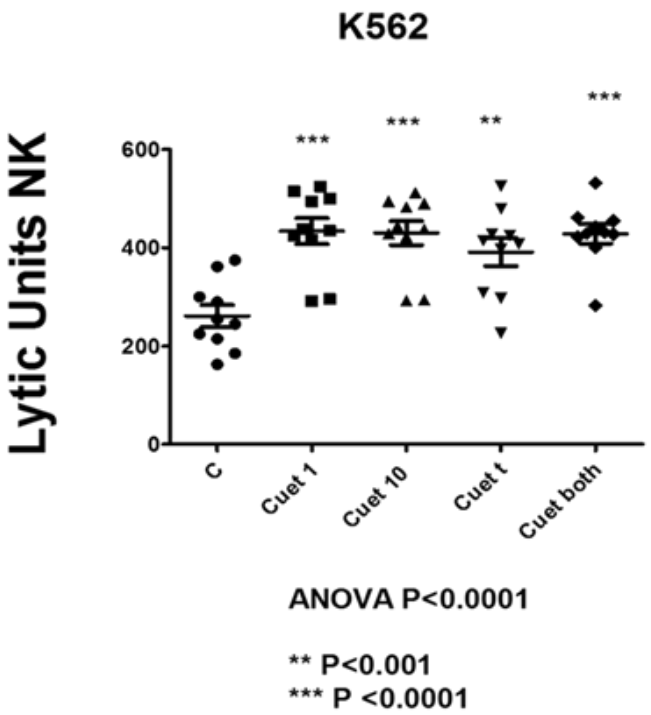
CuET treatment enhanced NK as well as T cell cytotoxicity against both K562 and K562 TAX cell lines. The K562 TAX cell line is partially resistant to NK spontaneous cytotoxicity; however, upon the treatment with CuET on lymphocytes, tumor cells or both, NK cells could efficiently kill them.

5.1.1 NK cytotoxicity assays

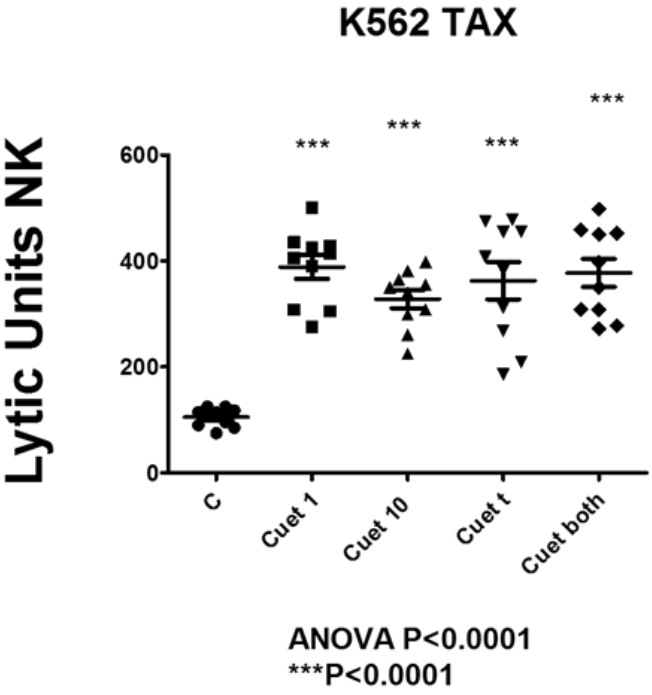
The CuET treatment evidently enhanced NK cell cytotoxicity against K562 cell line (Graph 1). No major difference was observed if the PBMCs, tumor cells or both were treated with CuET, except when only tumor cells were treated with 1 nM CuET the lytic units and the significance as compared to control was a little smaller.

Regarding the K562 TAX cell line, the increase in NK cell cytotoxicity was very significant, as can be seen in Graph 2. K562 Taxol resistant cell line is only partially susceptible to the immune response- it is resistant to NK spontaneous cytotoxicity, which is the reason why the control value (C) is so low. After the treatment with CuET there is a very significant increase in NK cytotoxicity not depending on whether PBMCs, tumor cells or both were treated with 1 nM CuET. As was already mentioned, when higher concentration s of CuET were used, the cytotoxicity was lower than the control. In some cases, lytic units obtained with 10 nM CuET stimulation were higher than the counterpart treated with 1 nM CuET.

The reason why the results represented in the Graphs 1 and 2 are dispersed as compared to T cell cytotoxicity (Graphs 3 and 4), is that the NK cytotoxicity is spontaneous, and depends on the number of cytotoxic NK cells of each donor. T cells are generated in standard culture conditions



Graph 1: NK cell cytotoxic activity against K562 cell line
C: control (no CuET was added to the samples). CuET 1 and 10: PBMCs treated with 1nM or10 nM CuET. CuET t: tumor cells treated with 1 nM CuET. CuET both: both tumor cells and PBMCs treated with 1nM CuET.



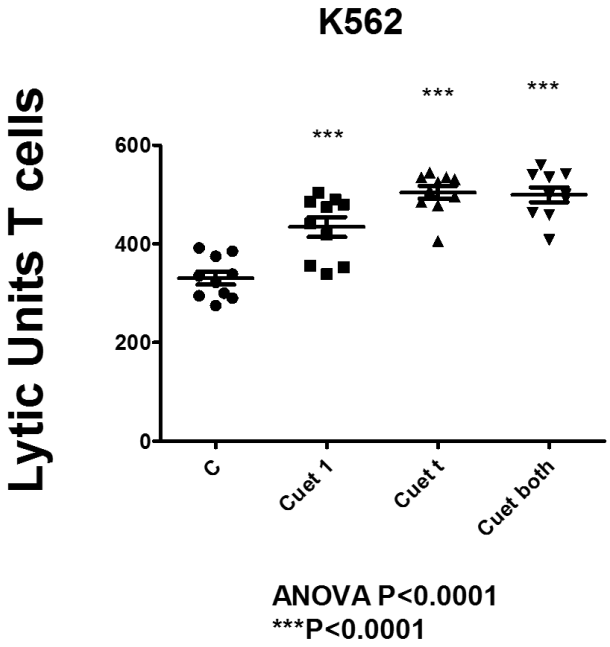
Graph 2: NK cell cytotoxic activity against K562 TAX cell line
C: control (no CuET was added to the samples). CuET 1 and 10: PBMCs treated with 1nM or10 nM CuET. CuET t: tumor cells treated with 1 nM CuET. CuET both: both tumor cells and PBMCs treated with 1nM CuET.

5.1.2 T cell cytotoxicity assays

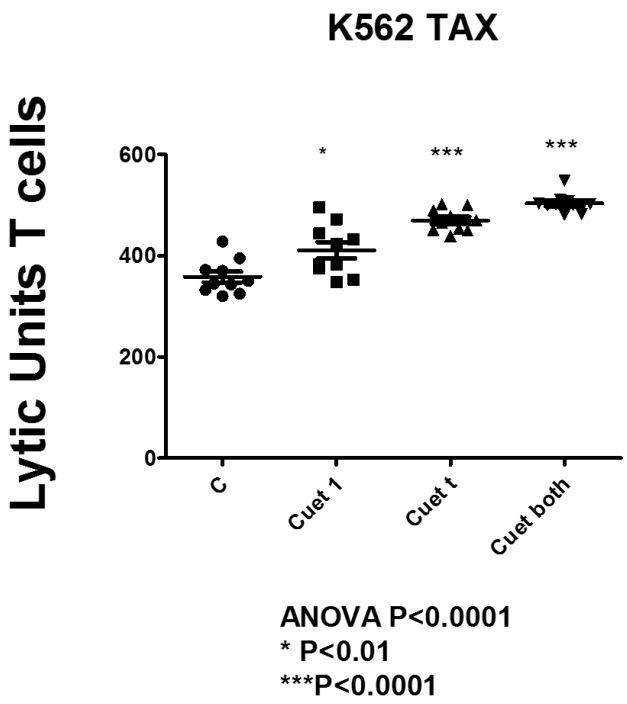
The T cell cytotoxicity represents specific immunity- antigen presentation is required, so the results are not as dispersed as for the NK cell cytotoxicity. As already mentioned for T cytotoxicity assays, only 1 nM CuET was used.

As can be seen in Graph 3, CuET treatment very significantly enhanced T cell cytotoxicity against the K562 cell line. No major difference was observed if the PBMCs, tumor cells or both were treated with 1 nM CuET.

Regarding the K562 TAX cell line, the increase in T cell cytotoxicity was very significant when tumor cells or both PBMCs and tumor cells were treated with 1 nM CuET. On the contrary, when only PBMCs were treated with 1 nM CuET the increase in T cell cytotoxicity was not very significant.



Graph 3: T cell cytotoxic activity against K562 cell line
C: control (no CuET was added to the samples). CuET 1: PBMCs treated with 1nM CuET. CuET t: tumor cells treated with 1 nM CuET. CuET both: both tumor cells and PBMCs treated with 1nM CuET.

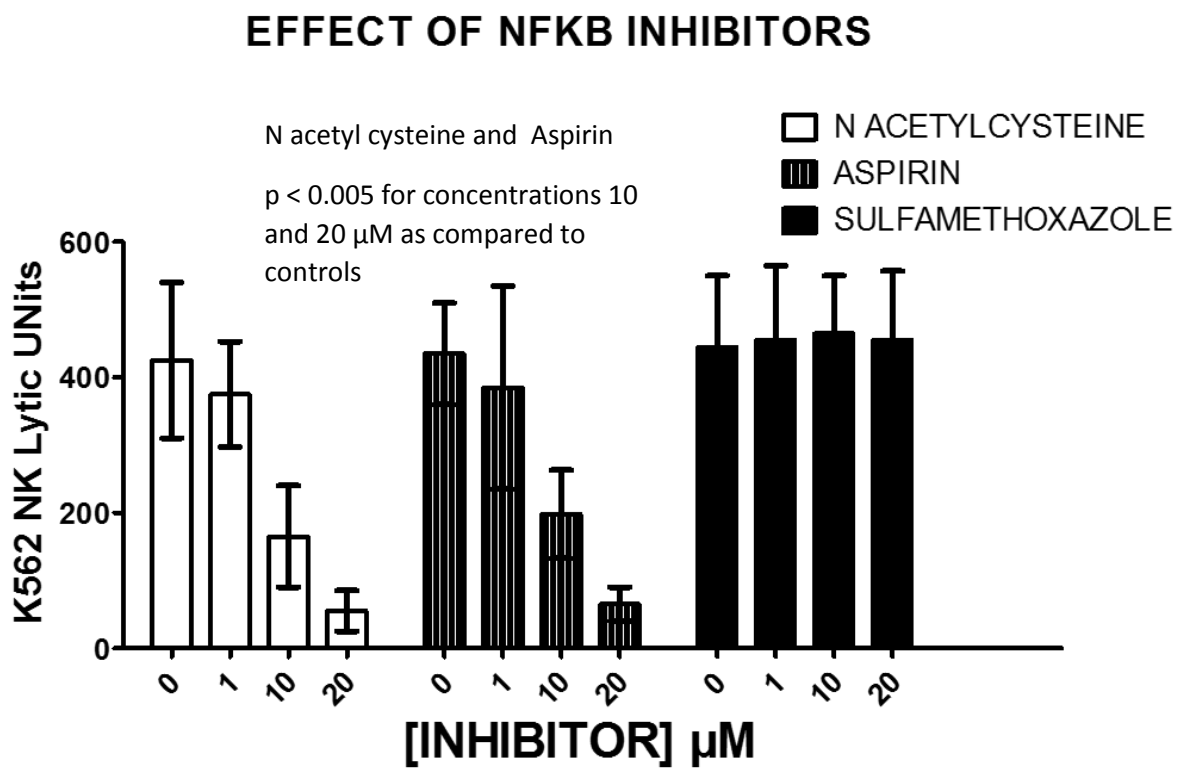


Graph 4: T cell cytotoxic activity against K562 TAX cell line. C: control (no CuET was added to the samples). CuET 1: PBMCs treated with 1nM CuET. CuET t: tumor cells treated with 1 nM CuET. CuET both: both tumor cells and PBMCs treated with 1nM CuET.

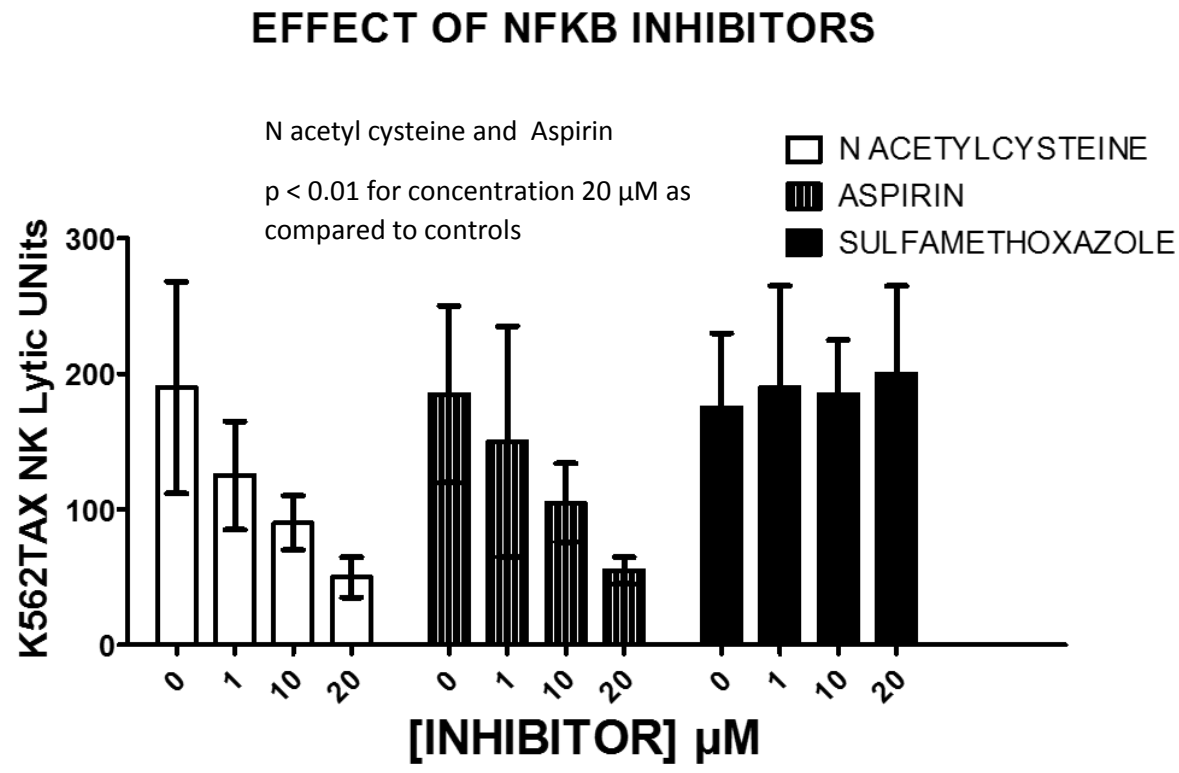
5.2 Effect of NF-kB inhibitors

One of the proposed mechanisms why there is an increase in NK and T cell cytotoxicity when the CuET treatment is used is the activation of NF-κB. Inhibition of NF-κB should abolish the effect of CuET on NK and T cytotoxic responses. NF-κB Inhibitors: N acetylcysteine and aspirin, sulfamethoxazole as control, were used for this experiment. PBMCs from 10 different blood donors were stimulated with 1nM CuET, preincubated in tubes with inhibitors at increasing concentrations: 1 μm, 10 μm, 20 μm for 30 minutes at 37° C. Then the cytotoxic assays were performed as described in Chapters 4.5.3 and 4.5.4.

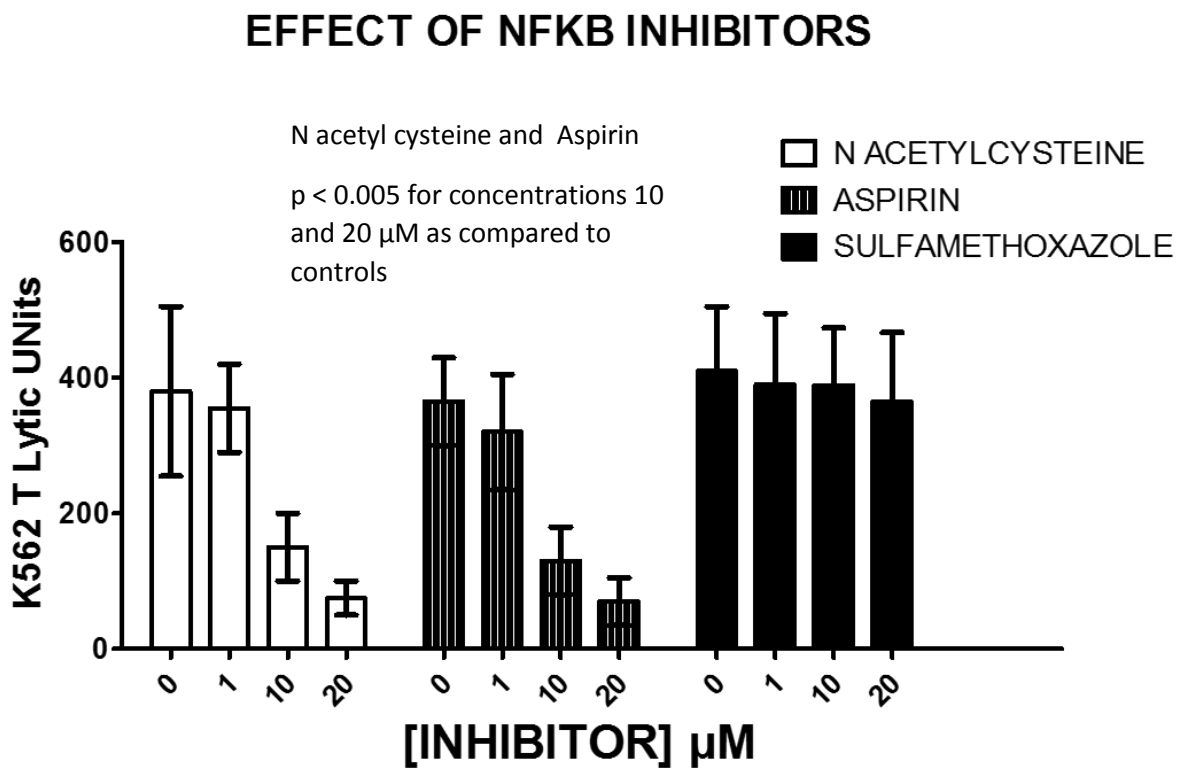
As can be seen in Graphs 5, 6, 7 and 8 the inhibition of NF-κB caused by N acetylcysteine and aspirin resulted in the decrease of T and NK cell cytotoxicity against K562 as well as K562 TAX cell lines. In all cases, the T and NK cell cytotoxicity was decreasing with increasing concentrations of the NF-κB inhibitors. On the other hand, sulfamethoxazole used as a negative control had no effect on the inhibition of NF-κB, so the CuET treatment was able to significantly enhance NK as well as T cell cytotoxic responses against K562 and K562 TAX cell lines. These results confirm that activation of NF-κB could be one of the mechanisms responsible for CuET cytotoxicity against cancer.



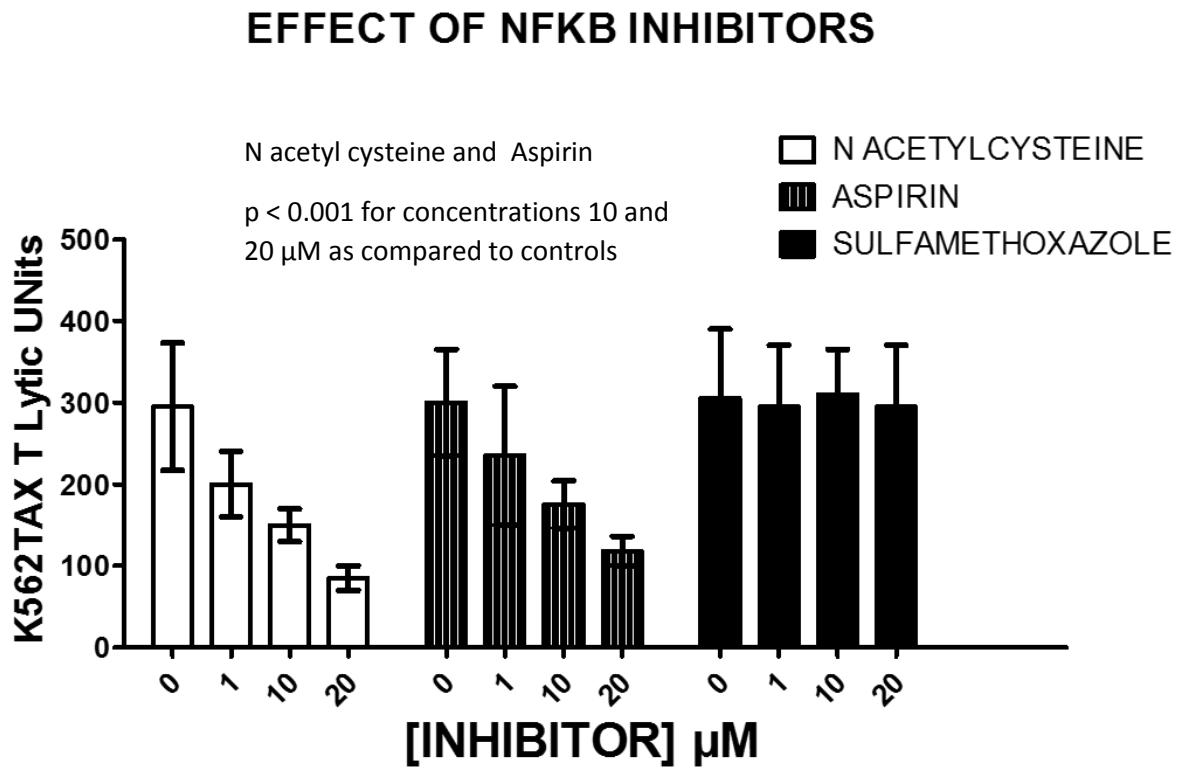
Graph 5: Effect of NF-κB inhibitors on NK cell cytotoxic activity against K562 cell line



Graph 6: Effect of NF-κB inhibitors on NK cell cytotoxic activity against K562 TAX cell line



Graph 7: Effect of NF-κB inhibitors on T cell cytotoxic activity against K562 cell line



Graph 8: Effect of NF-κB inhibitors on T cell cytotoxic activity against K562 TAX cell line

6 DISCUSSION

Disulfiram is an old drug that has been used to treat alcohol dependence for over 60 years (Jiao *et al*., 2016). However, disulfiram (DSF) was not only used to treat alcoholism. DSF was also shown to decrease cocaine consumption in cocaine-dependent patients (Gaval-Cruz *et* Weinshenker, 2009) and improved immunology parameters in patients with AIDS-related complex (Lang *et al.*, 1985; Lang *et al*., 1988; Hersh *et al*., 1991). However, more extensive clinical trials refuted previous conclusions (Vanham *et al.*,1993; THE HIV87 STUDY GROUP 1, 1993).

DSF anticancer activity was confirmed in many clinical trials and studies (Lewison, 1977; Dufour *et al.*, 1993; Askgaard *et al.*, 2014; Wickström *et al.*, 2007). Recently, Skrott and colleagues confirmed that for the anticancer activity of the drug is responsible its metabolite CuET (Skrott *et al*., 2017). The mechanism of action, however, has not been completely dilucidated. In 2006 DSF anticancer effects were related to its proteasome-inhibitory abilities (Chen *et al*., 2006) which was recently refuted by Skrott *et al*., 2017. The other mechanism of DSF, acetaldehyde dehydrogenase inhibition was also refuted by Skrott, *et al*., 2019. Promising possible mechanism of DSF anticancer activity was proposed by already mentioned Skrott *et al.*, 2017 indicating that DSF targets tumor via p97 segregase adapter NPL4 involved in folded protein catabolism. Since this discovery is very fresh, only further studies can show whether this mechanism will be confirmed or refuted. Skrott's experiments were done using oral DSF and oral copper gluconate in mice and CuET was found as the metabolite of DSF in the tumor cells.

In this theses, we introduced another possible mechanism of DSF anticancer activity. DSF, at 1 nM, was able to induce spontaneous and antigen-dependent cytotoxicity probably by the induction of cytotoxic receptors in effector cells and antigen expression in tumor cells. NF-κB enhances NK spontaneous cytotoxicity by the expression of cytotoxic receptors related to NF-κB activation (Kwon *et al*., 2016). We confirmed that CuET treatment enhances T cell cytotoxicity against K562 and K562 TAX cell lines. Inhibition of NF-κB by N acetylcysteine and aspirin abolished the effect of CuET on NK and T cell cytotoxic responses. The important finding is that CuET at 1 nM concentration is able to induce an increase in spontaneous cytotoxic response even against NK resistant cell lines. This increase may be due to an increased expression of cytotoxic receptors or an enhanced activation of the cells through NF-κB, future research should be performed on this issue. Interestingly, as it was previously discussed disulfiram inhibits NF-κB at high concentrations. *In vivo* experiments with CuET should solve the importance of NF-κB activating immune response and tumor growth.

We acknowledged that CuEt is a promising anticancer drug, and we hope that our results might contribute to its common use in cancer treatment. The challenge in *vivo* is to make this drug available in the bloodstream since the drug is usually dissolved in DMSO. Dr. Cvek has been trying to achieve this goal for several years (Cvek, 2012).

7 CONCLUSION

This thesis was focused on the effect of CuET on the cytotoxic response, which can be due to an increase in NK cell activity or antigen presentation, specific T lymphocyte cytotoxicity. Our results showed that CuET treatment enhances NK and T cell cytotoxicity against K562 as well as K562 TAX cell lines. One of the proposed mechanisms of the anticancer activity is the activation of NF-κB. We confirmed that the inhibition of NF-κB by its inhibitors N acetylcysteine and aspirin abolished the effect of CuET on NK and T cytotoxic responses. These results confirm that CuET is responsible for DSF anticancer activity and suggest that the activation of NF-κB could be the mechanism behind its anticancer activity.

The next step is to perform these experiments in *vivo* on mice which is already in progress, and further studies are required to confirm the mechanism of action *in vivo*.

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