UNIVERZITA PALACKÉHO V OLOMOUCI

Přírodovědecká fakulta

Katedra biochemie

Effect of disulfiram metabolites on NK and T cell cytotoxic activity against tumor resistant cell lines

BAKALÁŘSKÁ PRÁCE

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Studijní obor:		Biotechnologie a genové inženýrství
Forma studia:		Prezenční
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Rok:	:	2021

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Jméno a příjmení autora	Viktor Valentini
Název práce	Effect of disulfiram metabolites on NK and T cell cytotoxic activity against tumor resistant cell lines
Typ práce	Bakalářská
Pracoviště	IMTM-Institute of Molecular and Translational Medicine
Vedoucí práce	prof. Juan Bautista De Sanctis Ph.D.
Rok obhajoby práce	2021

Abstrakt:

Hlavním cílem této práce je prozkoumat efekty metabolitu disulfiramu na imunitní buňky a prozkoumat možné změny v prezentaci antigenů nádorovými buňkami samotnými. Nejprve tato práce shrne, co je rakovina v obecné rovině a poté zvláštní pozornost zaměříme na kolorektální karcinom. Vybrali jsme dvě buněčné linie kolorektálního karcinomu: HT29 a HCT116, které byly použity pro experimenty. Dále bylo diskutováno využití a známé efekty disulfiramu a jeho metabolitu CuEt. Mimo jiné byl také prozkoumán vztah mezi cytotoxicitou CuEt a inhibicí NF-κB.

V experimentální části této práce jsme se pokusili objasnit hlavní faktor který vede ke změnám v cytotoxicitě a to ať už by se jednalo o možnou změnu v antigenové prezentaci nádorových buněk, stimulaci imunitních buněk či o vyvolání obou procesů zároveň. Těmito experimenty bylo detekováno markantní zvýšení cytotoxicity, a to jak v T lymfocytech, tak i v NK buňkách bez ohledu na to, které buňky byly ošetřeny 1 nM CuEt. Dále bylo zjištěno, že inhibicí NF-κB cytotoxicita vůči nádorovým liniím velmi citelně klesá.

Počet stran	24
Jazyk	Anglický

Bibliographical identification

Autor's first name and surname	Viktor Valentini
Title	Effect of disulfiram metabolites on NK and T cell cytotoxic activity against tumor resistant cell lines
Type of thesis	Bachelor
Department	IMTM-Institute of Molecular and Translational Medicine
Supervisor	prof. Juan Bautista De Sanctis Ph.D.
The year of presentation	2021

Abstract:

This thesis's primary focus is to explore the effects of a disulfiram metabolite on immune cells and explore the possible changes in antigen presentation of the tumor cells themselves. Firstly, this thesis will summarize what cancer is, and specifically, we will focus on colorectal cancer. We chose two colorectal cancer cell lines: HT29 and HCT116, which will be used for the experiments. Then disulfiram and its metabolite CuEt will be discussed along with its known effects and uses. We also explored the involvement of CuEt's cytotoxic effect with NF-κB inhibition.

In the experimental part, we try to determine the main factor that leads to changes in cytotoxicity, be it a possible change of antigen presentation of the tumor cells, the stimulation of immune cells or both at once. We end up detecting a clear enhancement of cytotoxicity in both T and NK cells no matter what cells were treated with 1 nM of CuEt. We also find out that inhibiting NF-κB leads to decreased NK or T cell cytotoxicity towards these tumor lines.

Keywords	cancer, disulfiram metabolites, NK cytotoxicity, T cell cytotoxicity, NF-κB, CuEt
Number of pages	24
Language	English

Aknowledgment

First, I would like to thank my bachelor thesis supervisor, prof. Juan Bautista De Sanctis Ph.D., to whom I am very grateful for his never-ending patience and readiness to answer all of my many questions. He was of incredible help, even in the worst of times, a person who taught me and showed me a lot and without whom I wouldn't have been able to finish this thesis. I would also like to thank Janošťáková Anna M.Sc. for showing me how to work with tumor cell cultures properly. I am also very grateful to Frydrych Ivo Ph.D. for helping me understand flow cytometry.

Second, I want to acknowledge the financial support provided by the grant of the Ministry of Education, Youth and Sport, Czech Republic: Molecular and Cellular Clinical Approach to Healthy Ageing, ENOCH (European Regional Development Fund project No. CZ.02.1.01/0.0/0.0/16_019/0000868, IMTM #869/V19).

Last but not least, I would like to thank my whole family for standing by me and being very supportive of everything I ever did.

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ACT	Adoptive cell transfer
ALDH	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
BRCA 1,2	Breast cancer supressor gene 1 and 2
CAR	Chimeric antigen receptor
CHK1	Checkpoint kinase 1
CRC	Colorectal cancer
CuEt	Diethyldithiocarbamate copper
DCC	Deleted in colorectal carcinoma protein
DEAB	D-aminobenzaldehyde
DNA	Deoxyribonucleic Acid
DSF	Disulfiram
FBS	Fetal bovine serum
HLA	Human leukocyte antigen
ICD	Immunogenic cell death
IL-1	Interleukin 1
IKK	IκB kinase complex
JNK	c-Jun N-terminal kinase
KIR	Kill inhibitory receptor
Me-DTC-SO	S-methyl-N, N-diethyldithiocarbamate-sulfoxide
MHC-I	Major histocompatibility complex class 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NKT	Natural killer T cell
Nrf2	Nuclear factor erythroid 2–related factor 2
PD-1	Programmed cell death protein 1
PMN	Polymorphonuclear leukocytes
ROS	Reactive oxygen species
TNF	Tumor necrosis factor
UV	Ultraviolet light

1 Introduction

Colorectal cancer is one of the most common cancers in the world. Cancers may be defined depending on their mutation rate: hot (high mutation rate) or cold (low mutation rate). For example, the HT29 is a cold tumor cell line, and the HCT116 is a hot tumor cell line. A cold tumor is difficult for the immune system to detect and destroy since it does not present as many antigens as a hot tumor.

This thesis's primary focus is to explore the effects of disulfiram metabolites on immune cells and explore the possible change in antigen presentation of the tumor cells themselves. This research has been inspired by a study made by Zdenek Skrott *et. al*; published in 2017 that focused on the possible anti-cancer mechanism of disulfiram and CuEt. In this thesis, I chose to test the cytotoxicity of colorectal cancer cells specifically. Firstly, this thesis will summarize what cancer is, and specifically, we will focus on the cell lines HT29 and HC116, which will be used for the experiments. Then disulfiram and its metabolites, mainly CuEt will be discussed along with its known effects and uses. Lastly, the effect of CuEt on NF-κB signalling will be explored by inhibiting NF-κB and observing changes in cytotoxicity.

2 Theoretical part

2.1 Immune system

The immune system is a complex array of cells and humoral factors that protect the individual from infection, cancer and other events. Two main elements define immune cell responses: innate or no requirement of previous antigen presentation and adaptive immune response in which antigen presentation is a critical element of antibody generation, specific cytotoxic responses, and tolerance. The cells of the innate immune response involve monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes (PMN), natural killer (NK) cells, and several innate lymphocyte subpopulations (NKT, innate lymphoid cells, and γδ T-lymphocytes). Their function is to eliminate pathogens or unwanted or senescent cells; they perform this through specific receptors. The degradation of pathogens or cells by phagocytic cells gives rise to the presentation of antigen to T lymphocytes and B lymphocytes to generate specific antibodies or specific cytotoxic response. The production of antibodies aids innate immune response in pathogen recognition and effectiveness in antigen or pathogen clearance.

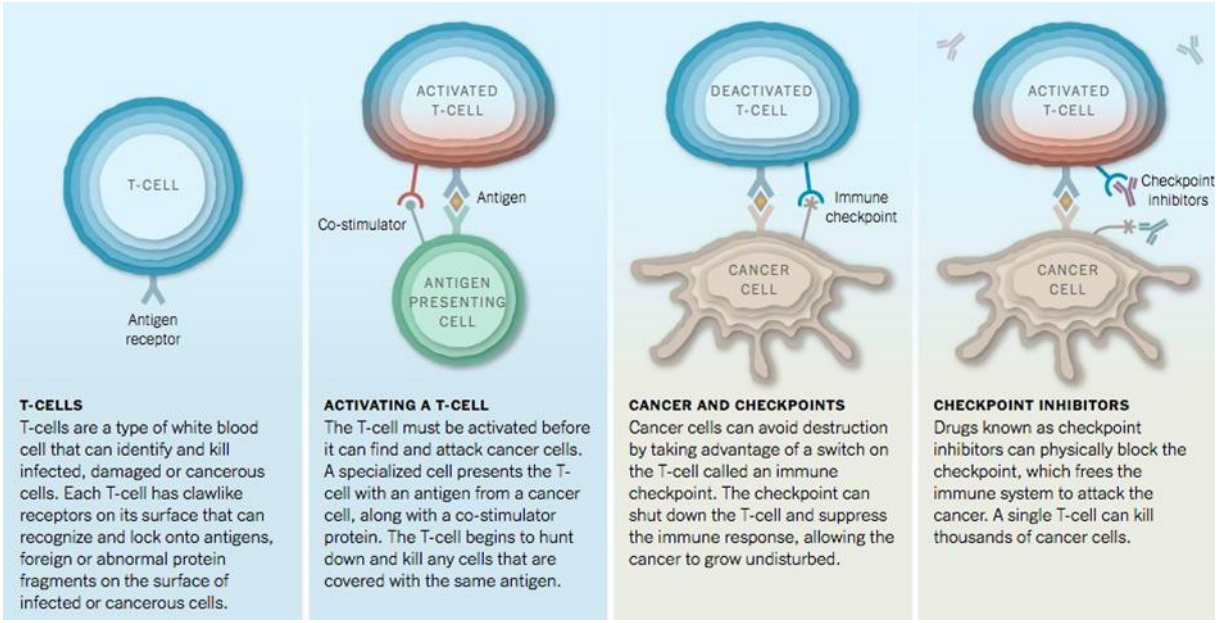


Fig. 1 - Mechanism of activation and deactivation of T-cells along with the mechanism of checkpoint inhibitor mediated T-cell cytotoxicity (Source: https://www.nytimes.com/2018/10/01/health/nobel-prize-medicine.html).[16.4.2021]

In cancer immune response, two key elements are required. Identifying specific antigens on the surface of tumor cells generates an immune response against it and the lack of inhibitory receptor ligands, mostly class I major histocompatibility antigens in the tumor surface. This mechanism of the MHC 1 complex deactivating the T-cell can be seen in fig. 1. The principle of immune checkpoint inhibitors like, for example, PD-1 and PD-L1 are also visualized. Three

cytotoxic mechanisms are essential in tumor elimination: 1) spontaneous cytotoxicity performed by NK cells recognizing cells with lack of MHC class one receptor and the presence of cytotoxic receptor ligands, 2) antibody-dependent cytotoxicity, which is dependent on cells expressing Fc receptors in the membrane, NK cells, macrophages, neutrophils, 3) antigen-specific T cell cytotoxic response. The immune response's efficiency in eliminating the tumor depends on antigen expression and tumor mutation. If the tumor rapidly mutates, it may escape both innate and adaptative immune responses. (Lichtman *et al.* 2017).

2.1.1 NK cell function and mechanism

Natural killer cells (NK cells)are a part of our innate immunity, but they also manifest some adaptive immunity responses. They constitute 5%-15% of circulating lymphocytes. They are found in several tissues, liver, uterus, etc., and are an essential part of our immune system since they eliminate virally infected cells or tumor cells. The major histocompatibility complex class mediates the mechanism of recognition of own (self) cells I (MHC class I) and other ligands that activate specific NK cell receptors involved in the cytotoxic response. Hence, if the NK cell recognizes a specific MHC-I binding to an inhibitory receptor, then no lysis of the target will happen, as shown in fig. 2a. If an NK cell fails to recognize the MHC-I with its KIR inhibitory receptor, or if the MHC-I is absent, then the NK cell will start a cytotoxic response towards the target cell shown in fig.2b. If a cell overexpresses induced stress ligands, then the MHC-I recognition mechanism will be overwritten, and the NK cell will engage its cytotoxic response (fig. 2c). Finally, antibody-dependent cytotoxicity will induce cell killing through activating Fc receptors on the NK cell membrane (fig. 2 d).

Cancer patients often do not have as many NK cells as healthy patients, or their NK cells are not responding to the stimulus. Transfer of NK cells has been an attractive treatment option for certain patients. There are two types of transfer: autologous and allogenic. Autologous involves isolating NK cells from the patients' peripheral bloodstream, cultivate and activate them *in vitro* before injecting them back into the patient. The allogenic transfer involves isolating NK cells from a healthy HLA matched donor and injecting them into the patient. The allogenic transfer has some advantages over autologous. NK cells of the donor may not recognize the MHC-I expressed on patients' cells by its KIR inhibitory receptors, resulting in a higher cytotoxic response towards tumor cells. Despite the mismatch of MHC-I, the donor NK cells don't end up destroying healthy cells, likely because of the lack of recognition of cognate antigens to activate NK cell receptors. (Morvan *et* Lanier, 2015; Shimasaki *et al.*,2016; Garber,2016)

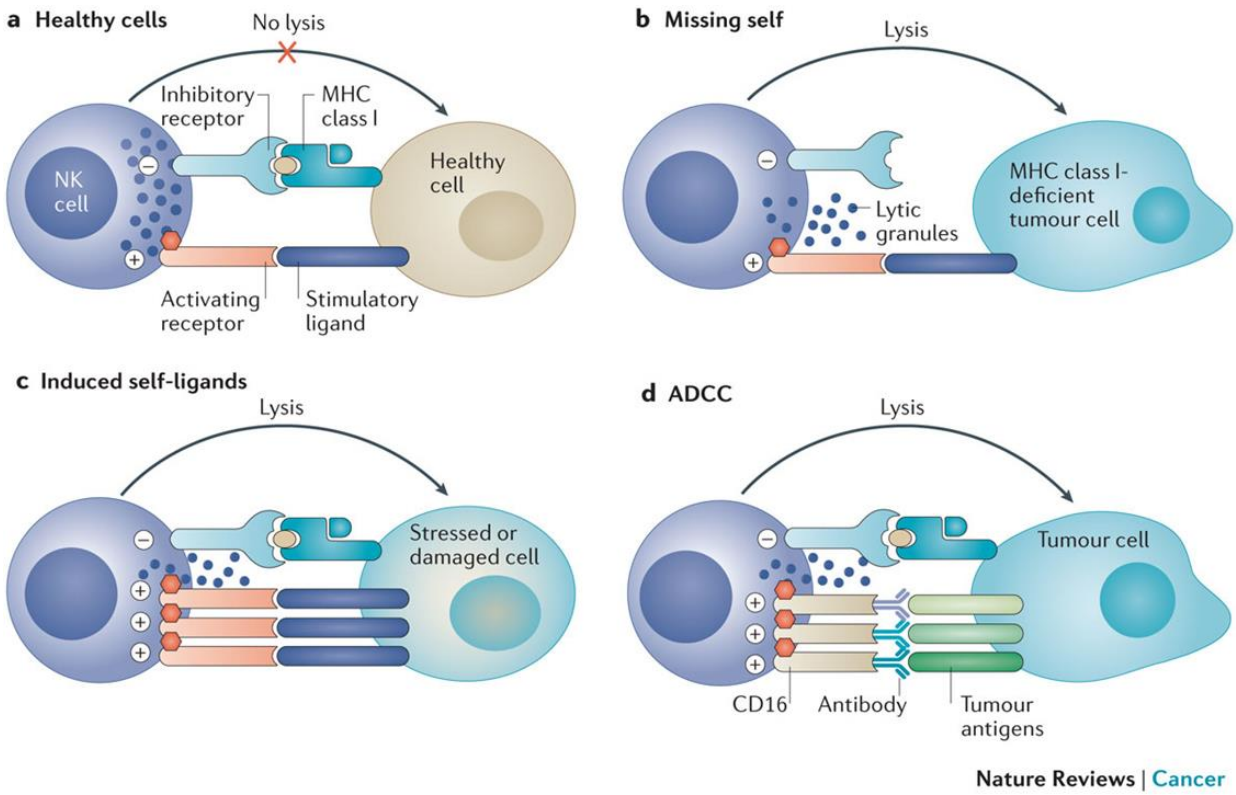


Fig. 2 – Mechanism of action of NK cells "a | A balance of signals delivered by activating and inhibitory receptors regulates the recognition of healthy cells by natural killer (NK) cells. b | Tumour cells that downregulate major histocompatibility complex (MHC) class I molecules are detected as 'missing self' and are lysed by NK cells. c | Tumour cells can overexpress induced stress ligands recognized by activating NK cell receptors, which override the inhibitory signals and elicit target cell lysis. d | Tumour antigen-specific antibodies bind to CD16 and elicit antibody-dependent NK cell-mediated cytotoxicity ". (From: Morvan *et* Lanier, *2016)*

2.1.2 Tumor heterogenicity

Tumor heterogeneity represents a major problem for the immune system (Truesdell *et al.* 2018). Several tumor clones and mutations upon treatment generate new epitopes, which may escape immune surveillance (O'Donnell *et al.* 2019). Also, metastasis, tumor invasion, and propagation involve the lack of antigen expression, characteristic of immature cells, and the uptake of antigens from normal cells of the invaded tissue. These autologous antigens generate tolerogenic immune responses. The enhancement of immune surveillance is, therefore, one of the aims of cancer therapy.

Several approaches have been used to enhance the immune response against tumor cells in numerous models (Cerezo-Wallis *et* Soengas, 2016, Cerezo *et al.*, 2015, Mebarki *et al.*, 2018, Benmebarek *et al.*, 2019, Heyman *et* Yang, 2019). One of the approaches is to use vaccines to enhance tumor surveillance (Cerezo-Wallis *et* Soengas 2016); however, tumor heterogeneity was a major drawback for its application (Truesdell et al. 2018). Another approach was to use *ex vivo* stimulation of lymphocytes using autologous antigen presentation. This process is

partially effective and very costly (Cerezo et al., 2015). Chimeric antigen receptors (CARs, also known as chimeric immune receptors) are receptor proteins that have been engineered to give T cells the ability to target a specific protein identified in the membrane of the tumor (Mebarki *et al.*, 2018). The receptors are chimeric because they combine both antigen-binding and T-cell activating functions into a single receptor (Benmebarek *et al.*, 2019). The use of different types of biological therapies that involved cytokines leads to promising results that finally lead to the inhibition of checkpoint inhibitors to enhance the immune response in cancer (Cerezo-Wallis *et* Soengas, 2016). Checkpoint inhibitors are composed of inhibitory receptors and ligands to decrease the inflammation burden (Szostak *et al.*, 2019, Alsaab *et al.*, 2017). Besides, pro-tolerogenic cells and permissive tumor microenvironment can be counteracted by this therapy. A decrease in tumor mass can be achieved, and consequently, patient's survival also increases. The treatment, nonetheless, cannot be continuously used and should be applied with concomitant therapy.

2.2 Cancer as an illness

Cancer is an abnormal and uncontrolled growth of cells of any organ or tissue in the body. A healthy cell becomes a cancer cell when it loses the ability to control cell growth and death. This lack of control often happens in the human body, but cell transformation usually does not occur. The immune cells identify and destroy the newly transformed cell before becoming cancer. Therefore the risk of developing cancer is higher in those persons whose immune system is suppressed. Several factors are involved in immune dysfunction, unbalanced nutrition, UV exposure, chronic stress, old age, previous chemotherapy, and abuse of drugs such as analgesics, antibiotics, and corticosteroids. (Roy *et* Saikia, 2016).

Currently, one in four people has a risk of developing cancer in their lifetime. The incidence of cancer in the world population keeps rising. This increase is probably because of our lifestyle, increased life expectancy, and increased exposure to cancer-inducing factors, UV radiation, chemicals, and other stimuli.

2.2.1 Colorectal cancer

"Colorectal cancer (CRC) is one of the most common cancers worldwide, with between one and two million new cases being diagnosed every year, thus making CRC the third most common cancer and the fourth most common cause of cancer-related death, with 700,000 deaths per year, exceeded only by lung, liver and stomach cancers." (Mármol *et al*.,2017). Around 55% of all CRC cases were diagnosed in western countries, and 33% of CRC-related

deaths occurred in 2010. This statistics is probably due to adequate healthcare and early screening. (Mármol *et al*.,2017).

Colorectal cancer mutation origin can be split into three groups: sporadic, inherited and familial. Sporadic cancers are caused by a point mutation and only affects an individual cell and its descendants. Sporadic CRC accounts for approximately 70% of all CRC cases. Sporadic CRC follows a specific sequence of mutations. It begins by the mutation of the tumor suppressor gene adenomatous polyposis coli (APC), which triggers the formations of non-malignant adenomas (polyps) and subsequent alteration of TP53 and DCC, generating a malignant cell. Only 5% of CRC cases are inherited. An inherited CRC is one where one of the alleles is already mutated, so it only takes a mutation in the other allele to create the tumor cell. Approximately 25% of cases are familial, which is also caused by inherited mutations. However, they don't fit in any of the inherited cancer variants, so they have been given their own group. (Mármol *et al*.,2017).

2.2.2 Current treatment approaches

The most common cancer treatments, also called conventional, are surgery, chemotherapy, and radiotherapy. Those approaches are not highly specific in eliminating only the cancer cells, and they usually present numerous, serious side effects in some cases. Most chemotherapeutics induce not immune-mediated apoptosis, which can lead to cell escape. Some compounds cause immunogenic cell death (ICD). The compounds induce non-immunogenic cancer cells to present antigens that are then recognized as non-self by the immune system (You *et.al*,2019). Many innovative cancer treatment approaches are still in pre-clinical research or early clinical trials. A lot of the innovative approaches not only focus on anti-cancer effectivity but also on high targeting specificity. Targeted therapy and immunotherapy seems to be a promising field. Targeting only cancer cells would most likely be very mild side effects than conventional treatments like chemotherapy or radiotherapy. Some of these approaches include passive and active targeting. Passive targeting involves exploiting the enhanced permeability and retention of tumor tissues and using nanoparticles to deliver the drug to the target. Active targeting involves using ligands that bind specific sites with nanoparticles attached to them. (Pucci *et.al*, 2019)

One of the most promising immunotherapy approaches is adoptive cell transfer (ACT). Immune cells, namely T-lymphocytes (T-cells), are isolated directly from the affected patient. The T-cells with the highest anti-cancer activity are isolated and expanded *ex vivo*. They can

also be genetically engineered to express a chimaeric antigen receptor (CAR). This genetic modification will make them more effective at recognizing cancer antigens. The percentage of patients with an end-stage diagnose of acute lymphocytic leukaemia that made a full recovery after ACT treatment is 92%. (Carlotta Pucci *et.al*, 2019)

2.3 Disulfiram and its metabolites

Disulfiram (1-(Diethylthiocarbamoyldisulfanyl)-N, N-diethyl-methanethioamide), also known as Antabuse, is mainly known because of its aldehyde dehydrogenase (ALDH) inhibitory effects. Disulfiram is widely used in alcohol abuse treatments and has also been shown to have anti-cancer properties in numerous studies. This effect was first thought to be due to its ALDH inhibitory effects. Disulfiram itself doesn't have any ALDH inhibitory function; this can be proven by blocking DSF metabolism steps with inhibition of P450, leaving liver ALDH function unaffected. (Yourick JJ *et* Faiman MD, 1991). But some of its metabolites like S-methyl-N,Ndiethylthiocarbamate-sulfoxide (Me-DTC-SO) have an ALDH inhibitory function. So this ALDH inhibitory effect of some metabolites has been thought to be the cause of its tumor-suppressing responses. This effect has been disproven, and the primary DSF metabolite responsible for its anti-cancer effects has been identified as CuEt, a metabolite that didn't seem to inhibit ALDH function. Moreover, the molecular target of the tumor-suppressing effect of DSF metabolite is NPL4, an adaptor protein of the p97/VCP segregase complex. (Skrott *et al.*,2017). A later study compared the effect of different disulfiram metabolites: 1) S-methyl-N, N-diethyldithiocarbamate-sulfoxide (Me-DTC-SO), ALDH inhibitor, 2) bis(diethyldithiocarbamate)–copper (CuET), a tumor suppressor with no ALDH inhibitory function, and 3) D-aminobenzaldehyde (DEAB) a known ALDH inhibitor. The results of this study confirmed that ALDH-inhibitory activity is not responsible for tumor suppression. Consequently, the study identified CuEt as the metabolite responsible for this mechanism. (Skrott *et al.*,2019).

The DSF metabolite CuEt induces the ROS-JNK stress-related pathway while inhibiting NF-κB(nuclear factor κB) and Nrf2 (nuclear factor erythroid 2–related factor 2). This event occurs in a dose-dependent manner. (Bing Xu *et. al*, 2017). In this thesis, the concentrations of CuEt used are nM, so that the mechanism may differ from that of high doses.

A study published in 2020 by D. Majera *et al.* focused on analyzing if CuEt treatment evokes replication stress, and it could provide an extra sensitivity to BRCA1 and BRCA2 deficient tumors. CuEt induced cell stress by slowing the speed of DNA replication, similarly

as hydroxyurea or aphidicolin. Moreover, CuEt blocks the ATRIP-ATR-CHK1 pathway and induces cell death in BRCA1 and BRCA2 deficient cell lines. (Majera et al. 2020). For reference, CHK1 (checkpoint kinase 1) is a serine/threonine kinase that regulates cell cycle checkpoints following genotoxic stress.

2.3.1 Disulfiram effects on immune cells

There are no studies that I could find regarding direct stimulation of immune cells, namely NK and T-cells, by CuEt. CuEt was used to stimulate lymphocytes and tumor cells to compare the effect of the treated cells separately. The concentration used is achievable in vivo in patients treated with disulfiram. This concentration ensures immune cells viability. The aim is to explore if CuEt in nanomolar concentration affects the NK and T cell cytotoxic activity against HT29 and HCT116 tumor cells.

2.4 Nuclear factor κ B

Nuclear factor κ B (NF-κB) was discovered by David Baltimore and his group in 1986. The NF-κB family encompasses 5 DNA-binding proteins. These proteins can regulate both innate and adaptive immune responses. NF-κB can inhibit apoptosis, stimulate angiogenesis and metastasis and even accelerate cell proliferation. (Taniguchi *et* Karin, 2018)

For the activation of NF-κB, the inhibitors of NF-κB (IκB) need to be degraded by the IκB kinase complex (IKK). Several proinflammatory cytokines are produced by this transcription factor, for example, IL-1 or the tumor necrosis factor (TNF). After activation, NF-κB can influence the cell and its microenvironment in many different ways, as shown in Fig. 3. (Taniguchi *et* Karin, 2018).

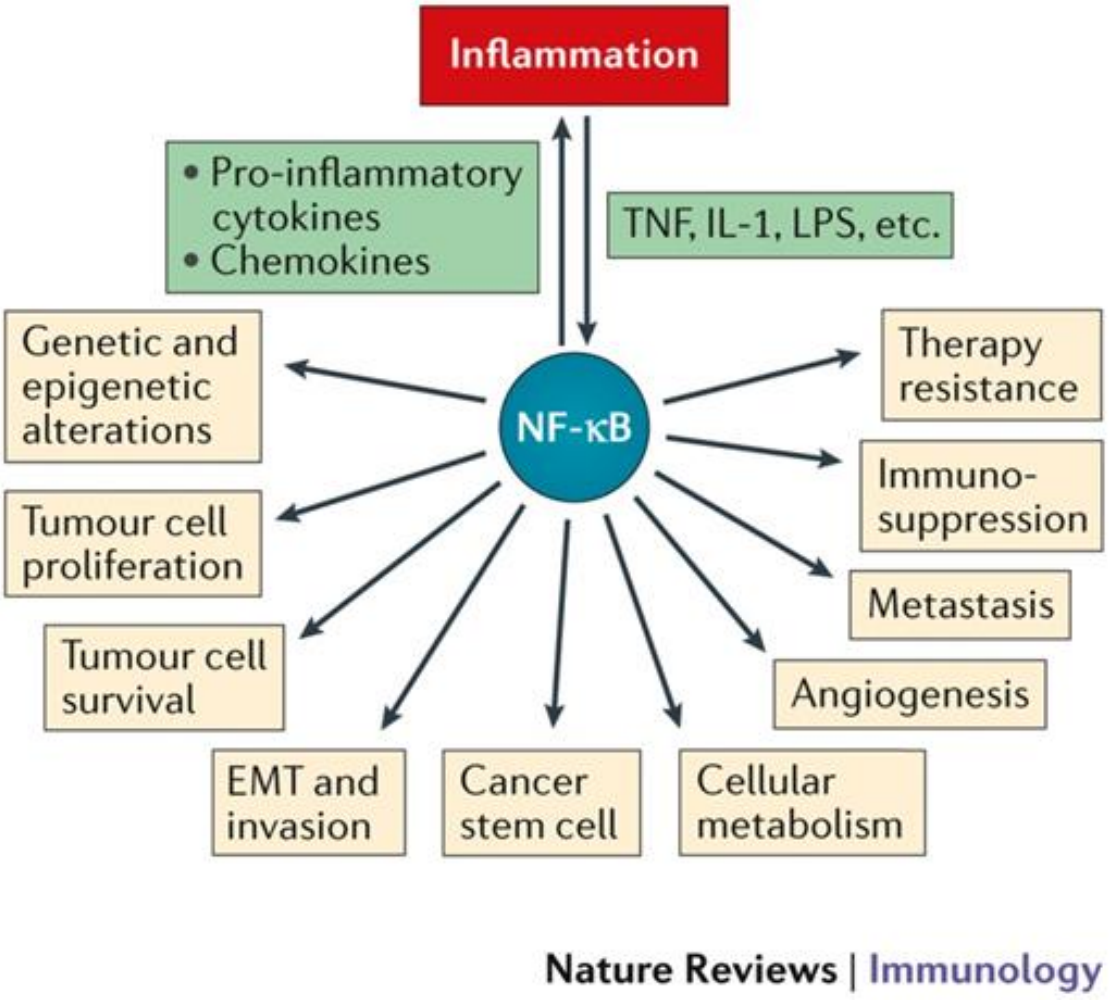


Fig. 3 – Ability of NF-κB to control many different processes - „Nuclear factor-κB (NF-κB) directly and indirectly controls inflammation, cancer cell proliferation and survival, epithelial-to-mesenchymal transition (EMT), invasive behaviour, angiogenesis and metastasis, as well as genetic and epigenetic alterations, cancer stem cell formation, cellular metabolism and therapy resistance. NF-κB activation also induces immunosuppression via several mechanisms. LPS, lipopolysaccharide; TNF, tumour necrosis factor." (Taniguchi et Karin, 2018) Picture from (Taniguchi et Karin, 2018)

3 MATERIALS AND METHODS

3.1 Biological material

The following cell lines were used for the experiments, HT-29 (ATCC® HTB-38™) and HCT 116 (ATCC® CCL-247™). Both were purchased from the American type tissue culture collection.

The cells were grown McCoy's 5a Medium Modified enriched by 10% Fetal Bovine Serum and antibiotics streptomycin (100 U·ml-1) and penicillin (100 U·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.

3.2 Chemicals and reagents

- 10% Fetal Bovine Serum (FBS) (Gibco, Cat. # 10270-106)
- McCoy Media Lonza Catalog Catalog #: 12-168F
- Aspirin (Sigma-Aldrich, Cat. # A2093)
- CuET (prepared by Dr. Petr Dzubak, Diethyldithiocarbamate (Sigma-Aldrich, Cat. # D3506) and Copper Chloride (Sigma-Aldrich, Cat. # 203149). Dissolved in dimethylsulfoxide (DMSO) final concentration of 2.6 mM
- 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)
- JSH 23 catalogue number 749886-87-1
- RPMI media with glutamine Lonza catalogue number BE12-702F

3.3 List of solutions

- 1x PBS: 10x PBS 80 g NaCl, 2 g KCl, 14,4 g Na2HPO4 · 2H2O, 2 g KH2PO4, dissolved in 800 ml dH20 and added to 1 l; preparation: mix 50 ml of 10x PBS + 450 ml dH20, filtrate before use using syringe filters
- Enriched RPMI medium (500 ml) for lymphocyte culture: Add 50 ml of 10% FBS and 50 ml of the antibiotic mix (streptomycin and penicillin, 100 U·ml-1) to 500 ml of RPMI medium, mix and filtrate before use using syringe filters
- McCoy media for tumor cells lines Add 50 ml of 10% FBS and 50 ml of the antibiotic mix (streptomycin and penicillin, 100 U·ml-1) to 500 ml of McCoy medium, mix and filtrate before use using syringe filters
- Trypsin use to detach tumour epithelial cells when confluent Thermo Fisher cat number 12604013

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)
- 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

3.5 Methods

3.5.1 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 to 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 75 cm^2 cultivation flasks in complete media 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.

3.5.2 Counting of the cells

Cell viability was determined by trypan blue exclusion using 90 μl of cells in the medium mixed with 10 μl of trypan blue. Then, 10 μl were pipetted into a hemocytometer from the prepared mixture, covered with a coverslip and placed under the microscope. Cells were counted in selected squares. The sum of counted cells was then multiplied by 10^5, representing the number of cells per ml. The cells were then adjusted to 2 million/ml by adding the complete media.

3.5.3 Cytotoxicity assays

Preparation of the cells for the assays

The CuEt was dissolved first in DMSO at a concentration of 2.5 mM and then diluted to complete media to 1 nM. PBMCs, 2 million cells/ml, were incubated in 6-well plates for 24 hours with 1 nM CuET. Tumor cell lines were incubated for 24 hours with 1 nM CuET at 2 million cells/ml. 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 nM CuET for 24 hr, and non treated tumor cells

3) non treated lymphocytes and 1nM CuET treated tumor cells

4) both effector and target cells treated with CuET 24 hrs prior the assay.

In all cases, the cell lines and the lymphocytes were washed twice with PBS before the cytotoxic assay.

NK cytotoxicity assay (spontaneous cytotoxicity)

Effector cells (PBMCs) were incubated with target cells (HT29/HCT1116) 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. Five thousand target tumour cells were used constantly in 100 μl of culture media per well. After the 4 hr 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 3.5.6).

3.5.4 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 to generate cytotoxic T cells against tumor cells and verify the specificity of antigens. After seven days, effector cells (PBMCs) were washed and then treated with 1 nM

CuEt for 24 hours or not treated. The cells were then rechallenged against live target cells (HT29/HCT1116) for NK cytotoxicity assay as described before.

Cross stimulation of mononuclear cells

To assess the specificity of cytotoxic response, mononuclear cells were stimulated with fixed HT29 cells but then challenged with HCT116 cells and vice versa.

3.5.5 Effect of NF-kB inhibitors

The NF-κB Inhibitors: N-acetyl cysteine and aspirin, and JSH-23 were used for this experiment. Sulfamethoxazole was used as a control. PBMCs from 6 different blood donors were stimulated with 1nM CuET for 24 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.

3.5.6 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 HT29/HCT1116 death was measured using a fluorimeter based on propidium iodide fluorescent 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.

3.5.7 Use of lytic units

The usual cytotoxicity analysis 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

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

However, since we have four effectors to target ratios for each treatment, 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

3.5.8 Statistical analysis

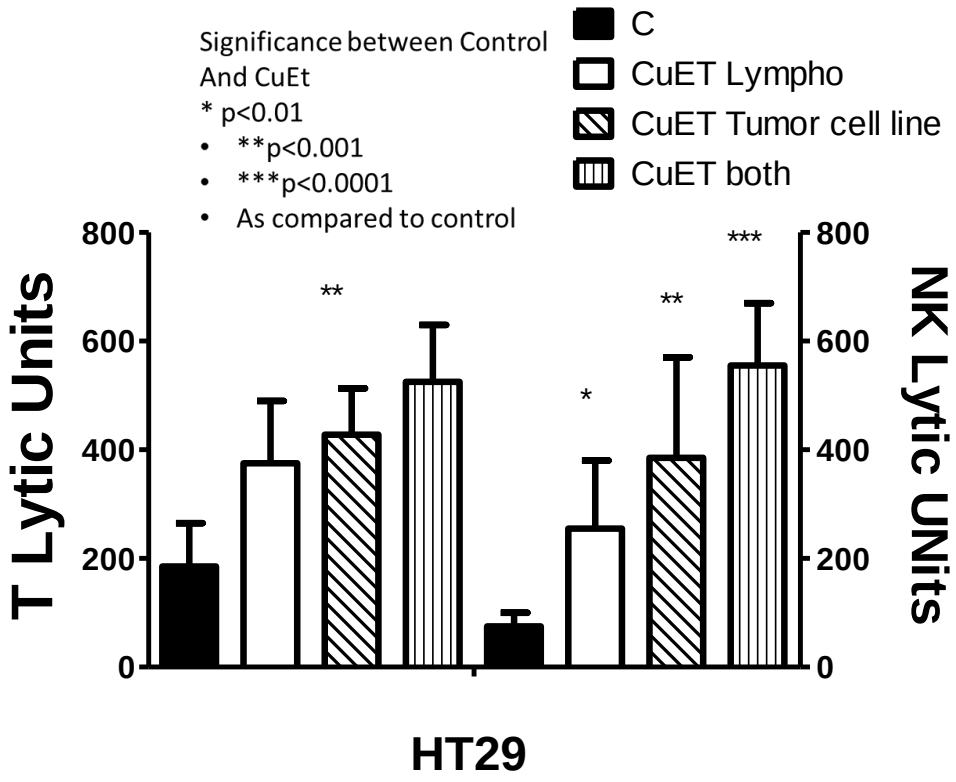
The statistical analysis of the results was performed in the GraphPad Prism 5 program (GraphPad Software, Inc.) using one-way analysis of variance (ANOVA), followed by Bonferroni t test for multiple comparisons.

4 RESULTS

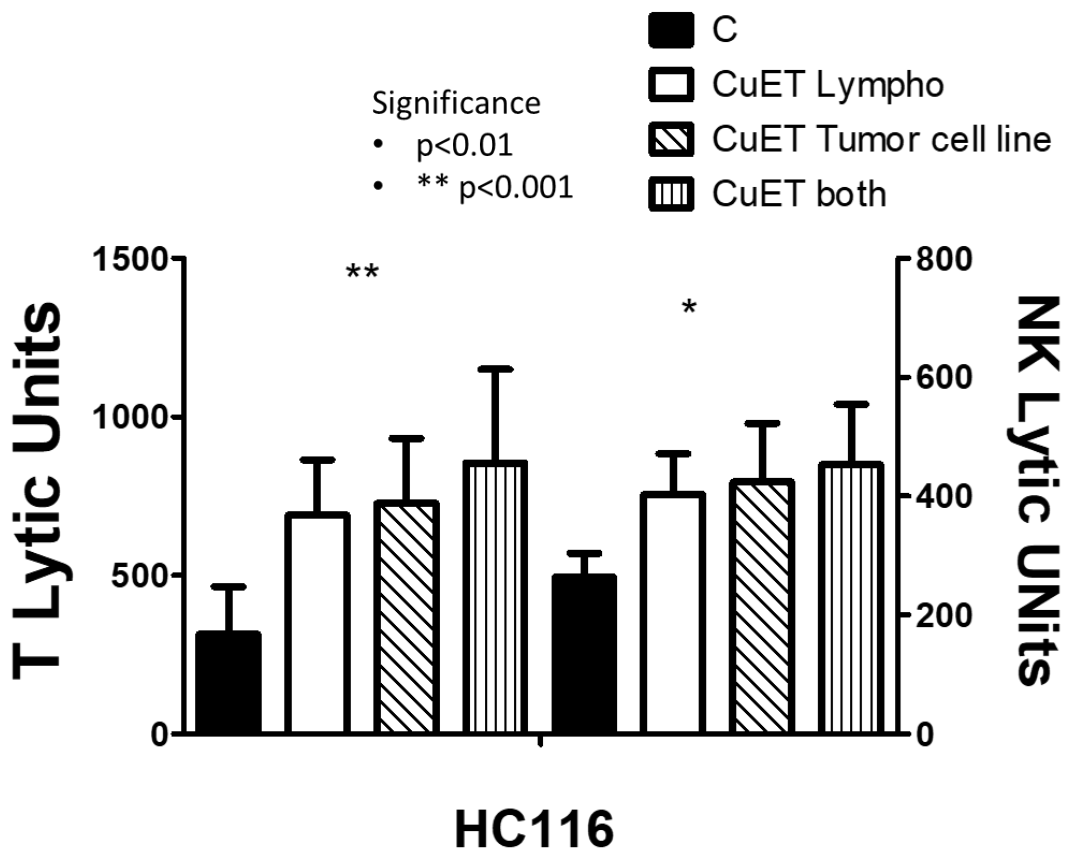
4.1 Cytotoxicity assays

Both HT29 and HCT116 cell lines have been treated and cytotoxicity measured as described in chapter 3.5.3. The data has been converted into lytic units described in chapter 3.5.7 and visualized in graphs 1 and 2. The results have shown an increase in NK spontaneous cytotoxicity after treating any of the cells with CuEt. The most significant difference in cytotoxicity happened between HT29 control (untreated) cells and both tumor cells and NK

cells treated with CuEt (Graph 1). The HT29 line being a cold tumor cell line that does not express antigens. The cytotoxic effect was more significant when immune cells were treated as opposed to the tumor cells being treated. In the HT29 cell line, we observed a significant increase in NK lytic units when both tumor and immune cells were treated as opposed to T lytic units or HCT116 lytic units for both NK and T-cells (Graph 2), showing only a small statistically insignificant increase when both were treated. The lytic units for the HCT116 cell line increased more when using T-cells rather than NK cells, with a statistically significant increase in lytic units when either one of the cells were treated (Graph 2).



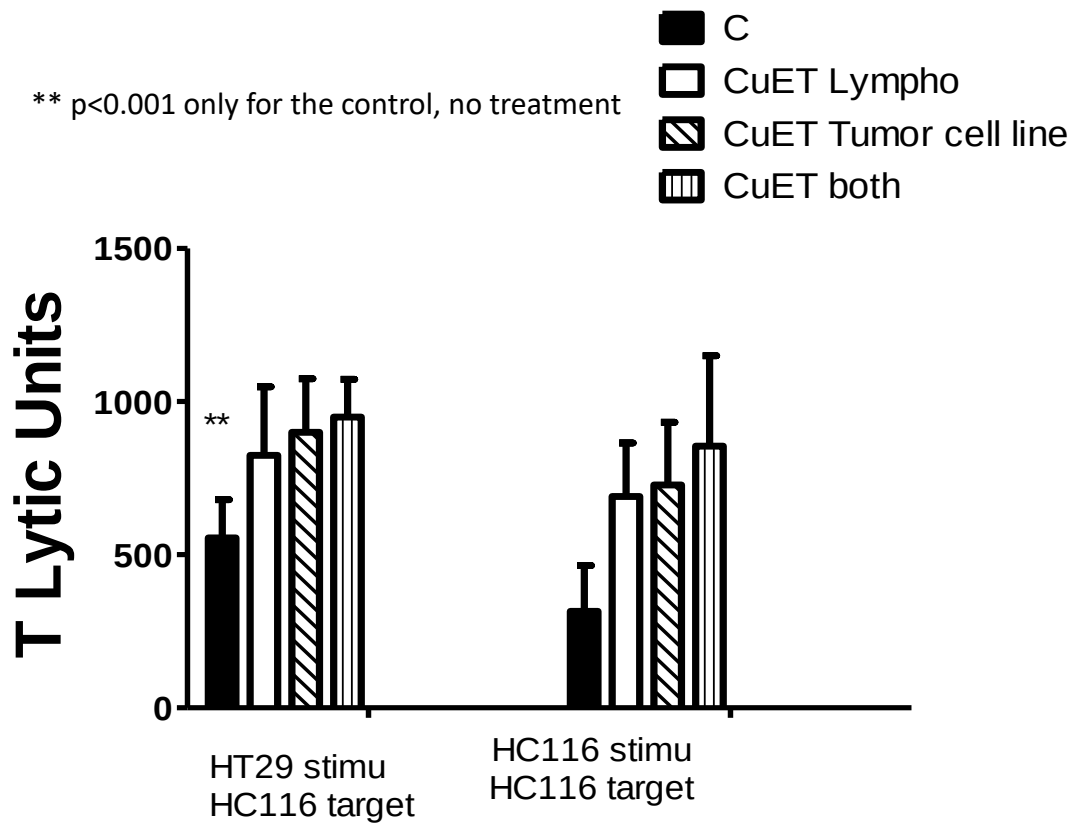
Graph 1 – Cytotoxicity assay towards HT29 cells in NK and T lytic units



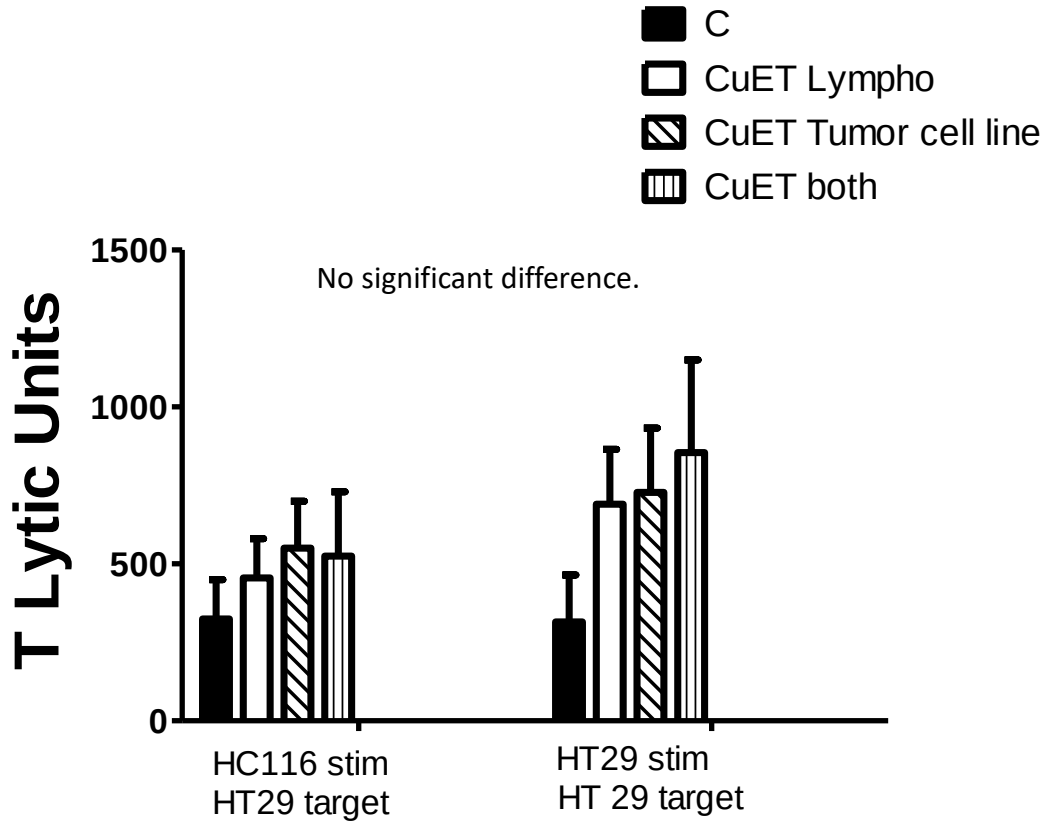
Graph 2 – Cytotoxicity assay towards HCT116 cells in NK and T lytic units

4.2 Cross stimulation cytotoxicity assays

Immune cells were stimulated with fixed HT29 cells but then presented with live HCT116 cells and vice versa cells, as described in chapter 3.5.3. Cytotoxicity was measured and converted into T lytic units. In graph 3, the untreated immune cells stimulated with the HT29 cell line clearly showed a statistically significant increase in cytotoxicity towards the HCT116 cells. However, when the immune cells were stimulated with HCT116 cells and presented with live HT29 cells, there was no effect (Graph 4).



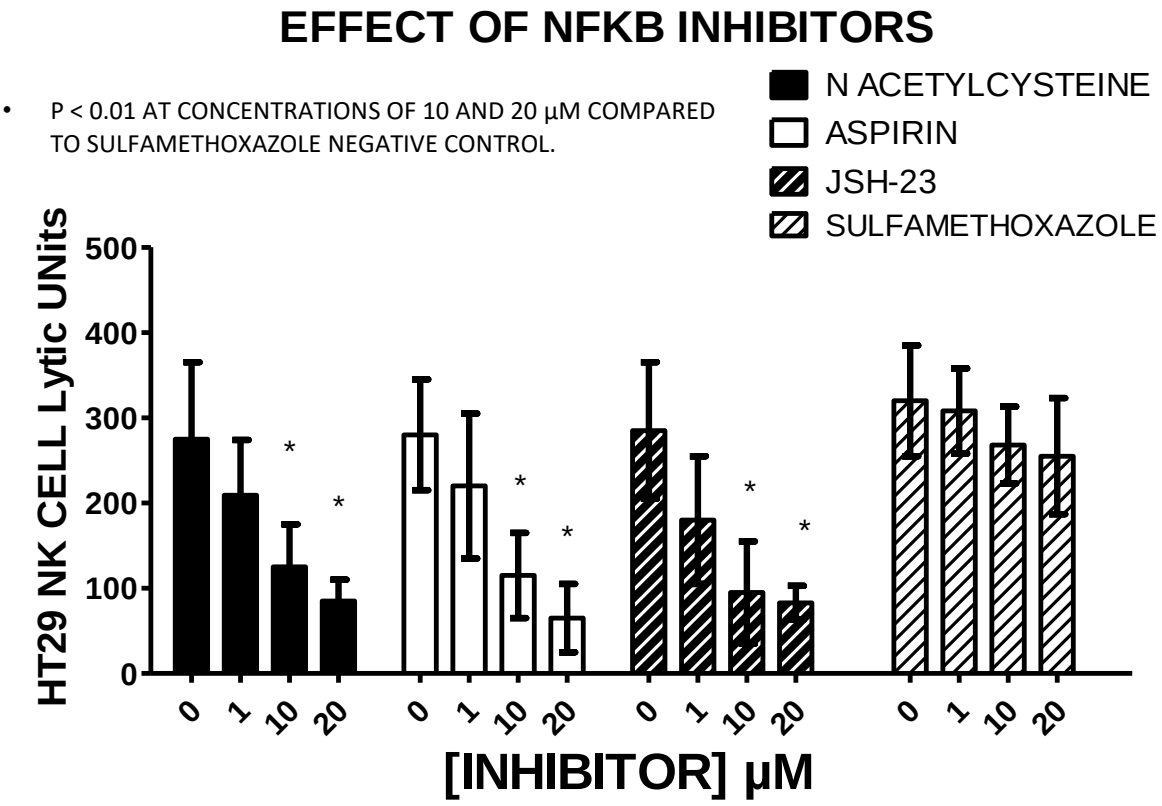
Graph 3 – Cross stimulation with HCT116 cells as target cells. HCT116 stimulated NK cells used as control.



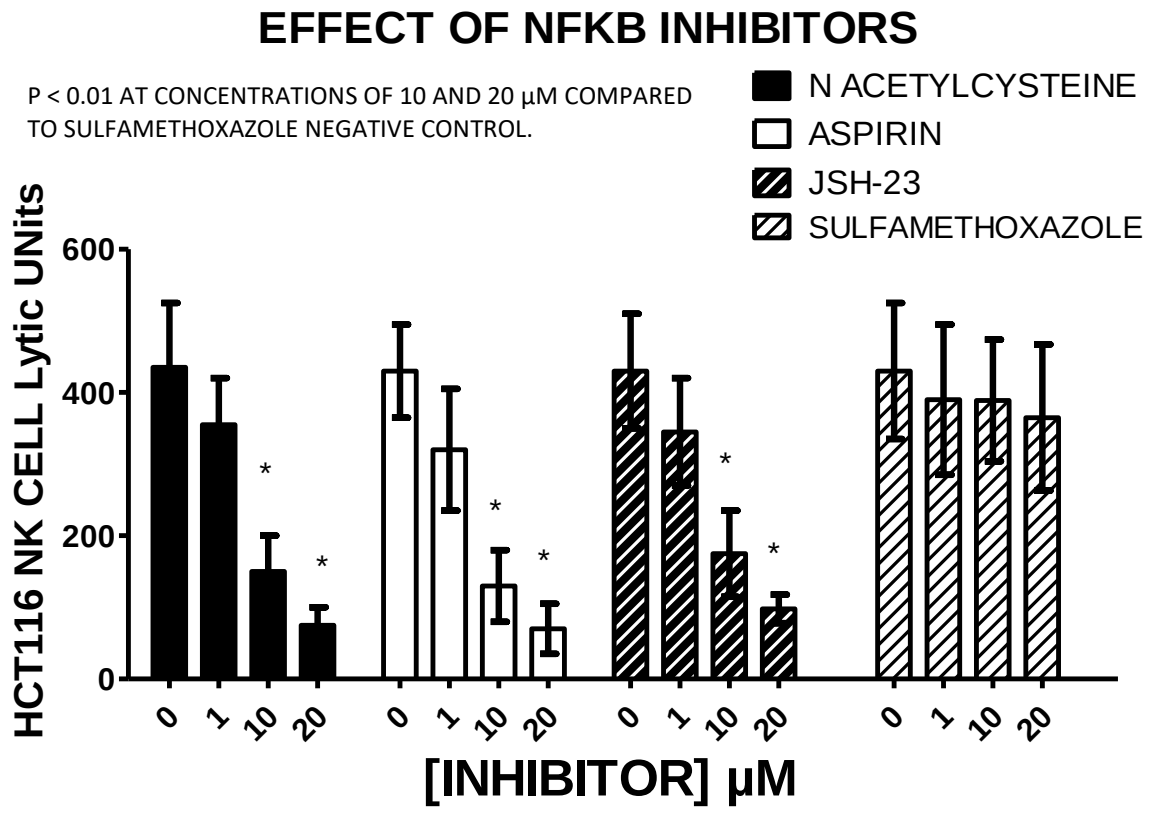
Graph 4 – Cross stimulation with HT29 cells as targe cellst. HT29 stimulated NK cells used as control.

4.3 Inhibition of CuEt effects on NK cytotoxicity

Both HCT116 and HT29 cells were stimulated with CuEt and preincubated with the inhibitor just as described in chapter 3.5.4. In both graph 5 and graph, 6 statistically a significant decrease in cytotoxicity was observed when at 10 and 20 µM concentration independently of the inhibitor used. The results were compared to sulfamethoxazole as the negative control. These results confirm that NFκB plays a crucial role in the anti-cancer effect of disulfiram if applied in 1 nM concentrations.



Graph 5 – Effect of NFκB inhibitors on HT29 cells sulfamethoxazole used as negative control. Inhibitors used for preincubation in 0, 1, 10 and 20 µM concentrations.



Graph 6 – Effect of NFκB inhibitors on HCT116 cells sulfamethoxazole used as negative control. Inhibitors used for preincubation in 0, 1, 10 and 20 µM concentrations.

5 DISCUSSION

After treatment with CuEt there has been a significant increase in cytotoxicity, especially NK cells against target cells (Graph 1,2). This effect may be due to a possible downregulation or inhibition of the PD1/PD-L1 checkpoint or the enhanced expression of cytotoxic receptors.

The NK lytic unit increase after CuEt stimulation was higher in the HT29 tumor cell line than HCT116. This effect is because the HT29 cell line doesn't express antigens on the cell surface, and CuEt promotes the expression of antigens in this cell line. On the contrary, on HCT116 cells, the cytotoxic response seemed to have reached a plateau. We propose that CuEt has a dual effect; the compound induces antigen expression on tumor cells and primes lymphocytes react against the tumor expressing antigens.

Regarding the cross stimulation, we observed an apparent increase of cytotoxicity in the untreated T cells stimulated with fixed HT29 and challenged with HCT116. This effect was not observed when the cells were stimulated with fixed HCT116 and challenged with live HT29 cells. The reason for this discrepancy can be because HT29 cells express specific antigens upon

CuEt treatment which are already expressed in HCT116. On the contrary, the variety of antigens expressed in HCT116 generate a broader cytotoxic T cell response, and consequently, only a subgroup of cytotoxic T cells will eliminate the HT29 cells.

The increase in cytotoxic response could be due to an increased transcription/secretion of interferon-gamma. More research needs to be done to confirm this conclusion.

The involvement of NFκB was investigated and was essential for the cytotoxic effect of CuEt. These results contrast previous studies (Bing Xu *et.al*, 2017) showing that CuEt inhibits NFκB. However, the experiments in those other studies performed in micro-molar concentrations of CuEt, whereas our experiments were performed at nano-molar concentrations. In bone healing, Copper decreases the inflammatory response and increases bone formation. The effect is dependent on NFκB and mitochondrial oxidative stress (Xu D. *et al.,* 2021). Probably, the responses on primary culture may be more informative than immortalized cell lines.

Notably, NFκB inhibitors had the same effect on both cell lines, confirming that the mechanism resulting in an increased susceptibility against NK and cytotoxic T cells is very similar for both cell lines. Further research is necessary to properly explore the mechanism of CuEt on NFκB at different concentrations.

6 Conclusion

Treatment of cells with 1 nM CuEt is non-toxic to tumor cells or lymphocytes. However, at this concentration, CuEt can prime HT29, HCT116, NK and T cells, making the tumor cells more susceptible and the lymphocytes more cytotoxic, resulting in increased tumor cell death.

The effect is inhibited by NF-κB inhibitors suggesting that the signalling pathway is important in lymphocyte cell activation. Cytokines, essentially IFNγ, could be involved in this process. Moreover, antigen expression in tumor cells seems to be increased upon treatment suggesting a dual effect, expression of antigens by the tumor and recognition by activated effector cells.

More research is required to study the mechanism of CuEt immune cell stimulation.

7 Literature

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