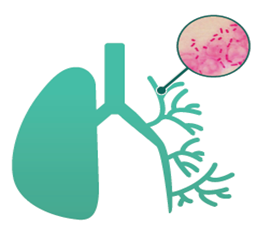
HZ UNIVERSITY OF APPLIED SCIENCES

Differentiation and reversal of malignant changes through application of novel chemicals

*Research report*

**24 January 2017**



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# SUMMARY

The thesis project was devoted to a differentiation of different types of cancer cells checking the effectiveness of the novel anti-leukemia chemicals and exploring whether the best anti-leukemia targets also target bone cancers.

The research question of this project is as follows: “How leukemia and osteosarcoma cancer cells can be differentiated in a most effective way and whether the novel anti-leukemia chemicals are also effective in osteosarcoma applying NBT/MTT screening procedures, cytotoxicity, morphological and alkaline phosphatase assays?”

In order to answer this question a set of the experiments has been executed. First, HL60 cell line was cultured and NBT/MTT test reflecting the level of maturation and viability was executed. The most efficient found chemicals, such as Dp-137 (10 µM), VC-82 (10 µM), NV-140 (10 µM), NV-139 (10 µM and 1 µM) have been subsequently tested with NBT/MTT assay using p-Nitro blue tetrazolium chloride and Thiazolyl blue tetrazolium bromide on K562 and KG1A cell lines. Afterwards, the centrifugation and Leishman’s staining followed by the morphology analysis was performed towards HL60, K562 and KG1A in order to confirm visually the level of maturation and viability. Then, the MTT experiments were carried out on healthy lymphocytes to identify their cytotoxicity effect. In conclusion, alkaline phosphatase using p-nitrophenyl phosphate and MTT cytotoxicity assays were carried out on osteosarcoma cell line to identify their effect on bone cancer cell line.

The obtained results showed that only Dp-137 with the concentration of 10 µM out of 26 screened chemicals causes the most efficient partial differentiation of HL60 (at the third day) and K562 particularly (at the third and the fifth days) with quite high cellular viability level. Nevertheless, Dp-137 did not show a significant efficacy towards KG1A or SaOS2 cell lines. Neither any above mentioned chemicals showed any differentiation effect on osteosarcoma cell line.

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# ABSTRACT

Nowadays cancer treatments usually involve the destruction of tumor cells. However, abnormal cancer cells may be persuaded to turn into normal cells through differentiation therapy applying less toxic substances. This work was devoted to leukemia and osteosarcoma cancers with the purpose to identify chemicals which would be equally effective for both cancer types since it could lead to the production of the medicine suitable for a wider circle of patients. NBT/MTT screening procedure following up with morphological, cytotoxicity and alkaline phosphatase assay have been carried out to identify whether the best anti-leukemia targets also target bone cancers. The chemical Dp-137 has shown the most efficient differentiation effect on leukemia HL60 and K562 cell lines. Neither any most favorable chemicals found for leukemia cell lines showed any potential differentiation towards osteosarcoma SaOS2 cell line.

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# INTRODUCTION

At the beginning of the 19th century approximately one in fifty deaths was cancer related. By the beginning of the 20th century it became one in twenty seven which is twice more frequent than in the previous century. After thirty years it had doubled again to one in twelve and by 1960 it became one in six. Taking into account statistics proposed in 1992 by the American Cancer Society, it was predicted that by the end of 20th century the situation would be even more dramatic and would lead to the number one in two. Based on UK Cancer Research, for the same period of time one in three people will develop cancer and that disease will lead to mortality of more than one in four (HMSO, 2003).

In fact, they were not too far from today’s reality. From head to toe, this disease has stirred up the destiny of many people regardless age and gender and according to World Health Organization it is considered to be one of the leading causes of morbidity and mortality worldwide (Stewart & Wild, 2014). The sad truth is that cancer is still a very long way from being defeated and nowadays, if someone is thinking of any type of cancer, most likely one considers death or a shortened life span. Unfortunately, this is especially true with some types of deadly cancer such as osteosarcoma and certain kinds of leukemia, particularly if prompt medical intervention is delayed.

In general, cancer enfolds more than 100 diseases developing over the time and enhances the division of the cells without control (National Cancer Institute, 2015). It begins when a cell skips from the normal restraint mechanisms of cell division and initiates its own program for proliferation. All subsequent cells created by division of the first cell, ancestral cell and the progeny reflect inappropriate proliferation as well. A tumor, which is a mass of cells, created from these anomalistic cells may remain in the tissue it was originated from (situ cancer) or it may initiate to affect nearby tissues (invasive cancer) (National Cancer Institute, 2007). However, the cancerous cells can occur in liquids as well, as in the case of leukemia. An invasive growth is considered to be malignant, and cells spread into the blood or lymph from an invasive tumor is most likely to create new cancer tumors (metastases) in the body. Tumors threaten a life when the growth destroys the organs crucial for survival (National Cancer Institute, 2007).

This research work will focus on two types of cancer, particularly leukemia and osteosarcoma, using cell lines originated from the source where these types of cancer take place. These types of cancer cannot be prevented as because they appear in a percentage of people, however timely and effective therapy could help to eliminate cancer’s severe consequences.

Regarding leukemia which is a type of blood cancer, it is usually disrupts the components of the white cells in the blood. It does not create a sufficient amount of them, so cancer patients are immunocompromised and becoming prone to infections. The multi-drug chemotherapy, stem-cell transplants and treatments with less severe side effects are making contributions to better survival from blood cancer. Nonetheless, some types of leukemia are still difficult to cure successfully, especially the ones which are more common in elderly people and adults (European Society for Medical Oncology, 2016).

In contrast, osteosarcoma is one of the most painful type of cancer is arising due to the solid malignant tumor which spreads in the bone tissues. Although osteosarcoma is considered to be a rare cancer, it is the most prevalent form of bone cancer and furthermore, it is the 8th most prevalent cancer among children and young adults (Union for International Cancer Control, 2014).

In this connection, taking into account this huge global problem, the project will be devoted to cancer research on leukemia and osteosarcoma cancer cells differentiation. Differentiation therapy considers cancer cells as normal cells, but with the lack of ability to control their differentiation – like stem cells stuck in a stem cell state. Usually cancer cells start to multiply before getting fully mature which leads to disturbances in the cell structure and shape. The sooner such cells start to divide, the more uncontrolled they become. Cancer with these quickly dividing cells will spread faster and is considered more aggressive. Differentiation therapy focuses on forcing the cancer cell to proceed the process of maturation applying pharmacological agents. So differentiated mature cancer cells resemble normal cells and tend to be less aggressive, grow and spread slower than undifferentiated cancer cells composed of immature cells. Differentiation agents are less toxic than traditional chemotherapeutic cancer treatments and do not kill so many cancer cells, however, they restrict the growth and permit the further application of chemotherapy to eliminate the cancer cells.

It is worth noting that some agents, such as *all-trans* retinoic acid (ATRA) have been identified to force certain type of leukemic cells to mature to the level where they no longer can cause cancer, however, nowadays these agents alone are considered not to be always effective, especially on a broad scale and in the long term. For this reason, they are often combined with chemotherapy. ATRA has also been used to some benefit with osteosarcomas and gliomas, even though not to curative effect. In this connection, there is a hope that some other chemicals will be identified which could induce differentiation of leukemia and osteosarcoma cells with better results. Furthermore, it is interesting to identify chemicals which would be equally effective for both leukemia and osteosarcoma cell lines due to the fact that it could lead to the production of the medicine which would be suitable for a wider circle of patients.

Thus, the novel innominate chemicals will be tested on leukemia cell lines from the labs of Greek colleagues, to check if they are effective in differentiating leukemia and/or osteosarcoma. NBT/MTT screening procedure which is developed in the laboratory of the University of Malta will be carried out, following up with morphological, cytotoxicity and alkaline phosphatase assay to identify whether the best anti-leukemia targets also target bone cancers.

Thus and so the main question is:

How leukemia and osteosarcoma cancer cells can be differentiated in a most effective way and whether the novel anti-leukemia chemicals are also effective in osteosarcoma applying NBT/MTT screening procedures, cytotoxicity, morphological and alkaline phosphatase assays?

During this project the following questions will be answered:

1. How to ensure good practices working with cell culturing?
2. What is the principle of NBT/MTT assay and how to interpret its results?
3. How to perform morphology analysis with regard to leukemia cancer cell lines?
4. Whether the differentiation chemicals used in the experiments cytotoxic with regard to healthy lymphocytes or not?

The main aim of the project is to perform a differentiation of different types of cancer cells checking the effectiveness of the novel anti-leukemia chemicals and to explore whether the best anti-leukemia targets also target bone cancers applying NBT/MTT screening procedures developed in the laboratory of Malta University, as well as morphological, cytotoxicity and alkaline phosphatase assays.

The project in which I am assigned is executed in the laboratories of the University of Malta as commissioned by Dr. Pierre Schembri-Wismayer, MD PhD.

The detailed overview of the experiments which are included in the section "Method" aims to support data to future study projects, support material for those, who want to reproduce the experiment.

# THEORETICAL BACKGROUND

The concept “cancer” is a common term for diseases that are caused by abnormal division of cells. The division of cells occurs when a cell duplicates and cleaves in two producing two daughter cells. Normal cells are aware of when to divide and when to stop in the contrast with cancer cells. It happens due to the fact that during DNA replication the genetic code of the cell is getting disrupted which leads to mutations and in some particular cases, to the deregulation. Such cell may be so damaged that the gene which is responsible for bringing the cell to its death by apoptosis is not able to function. Subsequently such cell is reproducing itself for many times to crowd other cells by forming a benign tumor.

However, in human bodies there are extremely effective defense mechanisms against external invasions. In the healthy organism, such mechanisms successfully deal with abnormal cancerous cells before they turn into tumors. Nonetheless, in some cases particular factors suppress the immune system making it too weaken to offer the resistance to the growth of cancer together with a malfunction of some cells.

Nowadays cancer treatments such as chemotherapy or radiotherapy usually involve the destruction of tumor cells (Liu & Yan, 2016). However, abnormal cancer cells may be persuaded to turn into normal cells through differentiation therapy applying less toxic agents than in a traditional chemotherapy with the aim to force cancer cells to continue the maturation activity and exclude tumor phenotypes.

The following subchapters are devoted to a more detailed overview of leukemia and osteosarcoma cancer nature with regard to cellular and molecular biology, cancer cells culturing peculiarities, differentiation technique and methods to evaluate its effectiveness, such as MTT/NBT assays, alkaline-phosphatase and morphology assays.

## 2.1 The cellular and molecular mechanisms of leukemia and osteosarcoma cancer cell behavior

Cancerous cells destroy the most fundamental principles of cell behavior which is the core of cell maintenance and arrangement. These breaches help to explore what the normal mechanisms are and in which way they are enforced. Thus, cancer research is focused on highlighting the fundamentals of cellular and molecular biology in order to understand them better and thereby, to understand cancer and tools to cure it. Thus and so, this subchapter focuses on the basic differences between healthy and cancer cells and the molecular mechanisms which trigger cancerous cell behavior with regard to leukemia and osteosarcoma.

Traditionally cancers are classified by the cell type or tissue from which they are formed. Thus, osteosarcoma which is one of the sarcoma subtypes arises from connective or muscle tissue forming the immature bone by neoplastic cells. Remaining cancers which do not arise from connective tissue or muscle cells include different leukemias (Alberts, Johnson, & Lewis, 2002). Despite the different nature of these cancer types, their affected cancer cells share some common underlying principles. Basically, all cancer cells in contrast with normal cells demonstrate six hallmarks needed to create malignant tumor. These principles were illuminated in “The Hallmarks of Cancer” which is the article published in the scientific journal “Cell” in 2000 by the researchers [Douglas Hanahan](https://en.wikipedia.org/wiki/Douglas_Hanahan) and [Robert Weinberg](https://en.wikipedia.org/wiki/Robert_Weinberg) (Douglas & Weinberg, 2000). These six hallmarks are the following:

1. *Continuous cell growth due to self-sufficiency in growth signals*

Usually cells of the body require hormones and certain molecules in order to grow and divide while cancer cells are growing without these growth signals. They have the ability to produce these signals by themselves (autocrine signalling). In addition, cancer cells can permanently “switch on” the signaling pathways to respond to these produced signals. Also, they can disrupt “off switches” which are playing role in the prevention of excessive growth which is stimulated by the signals (negative feedback) (Douglas & Weinberg, 2000).

1. *Insensitivity towards anti-growth signals*

Healthy cells control cell division and growth by proteins known as tumor suppressor genes. In case of DNA damage, these proteins prevent cellular growth and division, while in cancer cells they are altered, so cannot perform this function. Moreover, the healthy cells stop dividing when they occupy all the space around through the mechanism known as contact inhibition (McClatchey & Yap, 2012). Cancer cells do not have this ability which clearly can be seen by growing cancer cells in the plates. Such cells are keeping dividing and pile on each other in cloggy layers (Douglas & Weinberg, 2000).

1. *Infinitive amount of cell divisions.*

Cancer cells have “replicative immortality” which indicates that cancer cells can divide much more times compared with healthy cells. In fact, human cells may go through 40-60 rounds of cellular division with subsequent loss of the ability to divide (senescence), so they become “old” and finally die (crisis) (Bartlett, 2014). In the healthy cell DNA of telomeres (DNA sequence at the end of chromosomes) shortens after every cell division, subsequently becoming so short that it activates senescence and the cell stops dividing. In contrast, cancer cells can divide infinitively, avoiding senescence as because they produce an enzyme called [telomerase](https://www.khanacademy.org/science/biology/dna-as-the-genetic-material/dna-replication/a/telomeres-telomerase). Telomerase is a eukaryotic ribonucleoprotein (RNP) complex which stabilizes the length of telomers by adding TTAGGG repeats at their ends, thus avoiding the normal telomeres shortening during cellular division (Hiyama, Shay, Wright, & Zou, 2001).

1. *Skipping of programmed cell death.*

Cancer cells lose the ability for apoptosis even if the cells are becoming abnormal by avoiding the mechanisms which detect the damage. That leads to the absence of proper signaling which loses the ability to start the apoptosis. They can also have abnormalities in the downstream signaling as well, or in the certain proteins which will also prevent apoptosis (Douglas & Weinberg, 2000).

1. *Promotion of blood vessel development.*

New blood vessel development is obtained through the process called angiogenesis for the sake of oxygen and nutrients obtaining which plays a significant role in solid tumor development. Usually new blood vessels are produced during repair of the injuries or embryos development. By tricking these physiological processes, the tumors can expand themselves by producing new blood vessels in order to provide enough oxygen to cancer cells. To do this, cancer cells activate “angiogenic switch” which allow them to control healthy cells present in the tumor and have the ability to produce new vasculature (Douglas & Weinberg, 2000). The study performed at University of Florida College of Medicine showed, that in contrast with solid tumor, leukemia cells only recruit blood vessels but do not make contribution to the walls of the blood vessels (Hu, Jorgensen, Li, & Slayton, 2009).

1. *Invasion and formation of metastases.*

Cancer cells have the ability to spread into neighboring tissues by invading blood vessels, getting through the circulatory system with its specific harsh environment and by leaving this system they start metastasizing in the new tissue (Douglas & Weinberg, 2000).

The molecular basis for the development of osteosarcoma specifically is connected with the alterations of certain gene pathways, such as RB1, p53, DCC. Inactivation of both p53 and RB pathways is considered to be a central event in osteosarcoma cancer. P53 gene is involved in cell-cycle control, in copying with stressful cellular events, in checking cell proliferation in cases where it might be dangerous, thus, in [apoptosis](https://www.ncbi.nlm.nih.gov/books/n/mboc4/A4754/def-item/A4839/) and in protecting the organism against cellular breakage and death. This [tumor suppressor gene](https://www.ncbi.nlm.nih.gov/books/n/mboc4/A4754/def-item/A5906/) is altered in approximately half of all cancers in human body and when it is damaged, the altered cells do not always die, but rather continue to proliferate building up more genetic alterations through continuous cell division. P53 gene is located on 17p13 chromosome and its alteration may involve allelic loss, point mutations and gene rearrangements. Patients with Li-Fraumeni syndrome have a germline p53 mutation and therefore, a high risk to develop osteosarcoma. Moreover, abnormalities of the 18q22 region where DCC gene is located can be observed in the patients with Paget’s disease who have also a high risk for osteosarcoma (Capodano, 2003).

Acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are types of cancer which affect myeloblasts produced by myeloid stem cells. Myeloblasts are precursors to granulocytes which is a type of white blood cells with the function to destroy foreign objects. In myeloid leukemia cancer, a large amount of myeloid stem cells is turning into unhealthy granulocytes which are leukemic cells. These cells are getting accumulated in the blood and also in bone marrow, subsequently destroying the function of healthy red blood cells and platelets. These granulocytes do not combat infection as normal white cells do. These two types of leukemia are characterized by the differences in their hematological development. For example, in AML, myeloid cells are still reproducing and building up even if they do not mature properly, producing more unhealthy cells. Without a cure, the majority of patients only live for a few months. In contrast, in CML, the myeloid cells are close to full maturation but still do not mature completely, however, they look more morphologically healthy and therefore partly functional. It is known that 749 genetic aberrations are linked to AML and the four most common are t(15;17), t(6;11), t(8,21), Inv(16). All of the produced fusion proteins due to these aberrations disturb the process of cells maturation and differentiation and the regulation in signaling, cellular structure pathways and apoptosis, are disrupted.

Regarding CML, it is also considered to be as a hematopoietic stem cell disease, however it is caused by BCR-ABL translocation, which revealing itself in an active and uncontrollable protein tyrosine kinase. This gives hematopoietic stem cells the properties to proliferate in an immature form. The function of BCR is not fully comprehended, while ABL is known to act as a proto-oncogene. It encodes for a tyrosine kinase protein, which regulates cell differentiation process. Moreover, the translocation of the chromosomes 9 and 22 produces a mutated chromosome 22, called the Philadelphia Chromosome and encoding an active tyrosine kinase protein. In CML, uncontrolled growth of immature hematopoietic cells is partly due to BCR-ABL’s activation of Ras signaling pathway and the Ras activator. In addition, Bcl2 overexpression and JunB underexpression take place increasing continuous proliferation and survival in hematopoietic cells.

Although AML and CML are different types of cancer, leukemia related fusion proteins that disturb the process of hematopoietic differentiation create the molecular grounds for myeloid leukemia. This interference creates immature myeloid cells that builds up in the bone marrow, as well as in blood stream, causes some of the common myeloid leukemia symptoms (Reeder, 2014).

## 2.2 Cancer cells culturing techniques

Nowadays cell culturing is used extensively all over the world. The techniques allowing different types of cells to be grown outside the body have been developed during the 20th century and although a large caution is needed in culturing cells and in interpreting information received by examining cells in vitro, it has given a good possibility to investigate complex illnesses such as cancer by simplifying it to its componential parts (Langdon, 2004). The aim of this subchapter is to provide overview of some of the basic concepts practicing cancer cell culturing, focusing on aseptic techniques, possible culture contaminations, favorable culture conditions, sub-culturing, cell counting, as well as the overview of the cell lines which are going to be used in the experiments.

Basically, cancer cell culturing refers to the cells removal from human with their subsequent growth and maintenance in appropriate artificial conditions. The table below represents the overview of the leukemia and osteosarcoma cell lines which are going to be used in the project. All of the leukemia cell lines represent the morphology of lymphoblasts which are spherical in shape cells growing in suspension without being attached to a surface, while osteosarcoma cell lines represent fibroblastic morphology having elongated shapes and growing being attached to a substrate (Gibco, 2010).

*Table 1: The overview of leukemia and osteosarcoma cell lines used in the project*

|  |  |  |
| --- | --- | --- |
| **Cell name** | **Cell morphology and origin** | **Disease** |
| ***HL60*** | Lymphoblast in peripheral blood | *Acute promyelocytic leukemia (APL).* Sub-type M2 of AML without the chromosome translocation t15:17 |
| ***KG1A*** | Lymphoblast in bone marrow | *Acute myeloid leukemia (AML)*. Sub-type M2 with much more earlier blast formation and without the chromosome translocation t15:17 |
| ***K562*** | Lymphoblast in pleural effusion | *Chronic myeloid leukemia (CML)*  in blast crisis |
| ***SaOS2*** | Fibroblast in bone | *Osteosarcoma* |

In order to perform successful cell culturing, a variety of requirements should be met. The following key points described below present themselves as the core of cell culturing practices.

***Favorable culture medium and physical environment***

Culture conditions may vary for every cell type, however a suitable cell culturing vessel with a media should always contain such constituents as essential nutrients (minerals, salts, amino acids, vitamins, carbohydrates), hormones, growth factors.

For the majority of mammalian cells, the favorable temperature is around 36.5° ± 1°C. Culture media should contain a CO2 and usually 5% CO2 is preferred. The majority of cancer cells require a pH around 7.2–7.4. In addition, medium should be changed regularly, because cellular growth produces byproducts formed due to respiratory processes that lead to acidification of the media. Usually the pH indicator phenol red is added to the medium to ensure a visual indication of the pH. It is getting yellow when pH is 6.5, orange when pH is 7.0, red when pH is 7.4 and purple when pH is 7.8. Also, humidity levels are significant because the water evaporation from the medium causes overconcentration of salts which eventually will lead to cell lysis.

***Aseptic techniques and the prevention of contamination***

Successful cell culture requires the cells deprived of any kind of contamination, such as mycoplasma, other bacteria, viruses, fungi and cross-contamination with other cells from outside. Therefore, it is important to provide a contamination-free environment by sticking to aseptic techniques and good laboratory practices. Antibiotics can be added to the medium to avoid bacterial infection, however they do not prevent infections with fungus, viruses or mycoplasma. In order to avoid cellular contamination, it is compulsory to use an individual bottle of medium for every cell line and handle only one cell line at the same time.

Also, it is mandatory to use the cell culture hood using the UV light in order to sterilize the air and work surfaces between uses by switching it on for 30 minutes. The working surface should contain only items needed for a particular procedure, moreover they have to be wiped with 70% ethanol before placing them in the hood. The ethanol should be also used during work, particularly after any spillage. The cell culture hood should run all the time during the procedure and has to be switched off only when it will not be used for prolonged period of time. The hands should be washed before and after working with cell cultures and personal protective equipment has to be worn. Any reagents and glassware have to be always sterilized using autoclaving and sterile filtering. Sterile flasks or bottles should not be opened until is time to use it instantly. They also should not be left opened to the environment, so the cover has to be returned as soon as possible (Thermo Fisher Scientific Inc., 2015).

***Sub-culturing***

Sub-culturing gives the possibility to increase the number of cells achieving a higher growth fraction for the characterization, freezing preservation and further experimentation. The principle of the sub-culturing involves the separation of the cells from the substrate (in case with the fibroblasts) and from each other in order to produce a cell suspension that can be quantified. Transferring this cell suspension to reduce its concentration into a flask produces a secondary culture. It can be grown up and then sub-cultured again to produce a tertiary culture (Gibco, 2010).

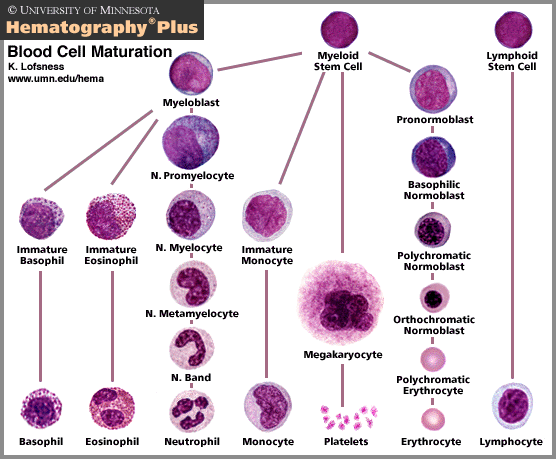
***Cell Counting***

The simplest way to count the cells involves the use of a hemocytometer which is a device for visual quantification of the number of cells in a fluid sample under a microscope. Basically, it consists of a glass chamber and 0.1 mm of quartz cover slip placed above the chamber floor. The quantification of cell number is performed by counting the cellular number inside a defined field underneath the cover slip (Langdon, 2004).

## 2.3 Haematopoietic and bone cell maturation and differentiation therapy as a promising method for cancer cure

[Haematopoietic stem cells](https://en.wikipedia.org/wiki/Hematopoietic_stem_cell) are located in [bone marrow](https://en.wikipedia.org/wiki/Bone_marrow) and give rise to myeloid and lymphoid stem cells and subsequently to all of the mature blood cell types, including red blood cells (erythrocytes), platelets and white blood cells. White blood cells include three types – granulocytes (neutrophils, eosinophils, basophils), monocytes and lymphocytes (Birbrair & Frenette, 2016). The chart below illustrates the blood cell maturation process. Cells start from “parent” myeloid or lymphoid stem cells continuing to specialize and mature.

The last row of the chart shows the most mature form of the blood cells.



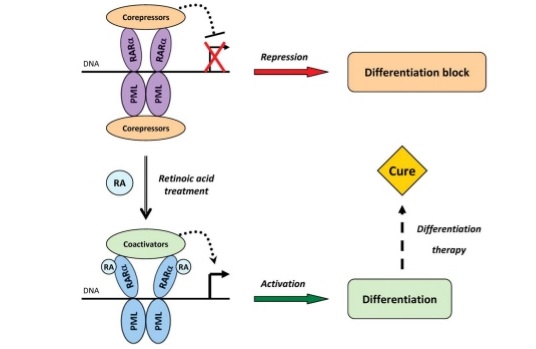
*Figure 1: Blood cell maturation chart* (University of Minnesota, 2008)

APL (corresponding HL60 cell line) is a subtype of acute myeloid leukemia, a white blood cells’ cancer where an abnormal accumulation of immature promyelocytes without further maturation takes place. AML (corresponding KG1A cell line) is a type of cancer where usually an abnormal accumulation of myeloblasts takes place without further maturation, however it can also affect red blood cells and platelets. CML (corresponding K562 cell line) is a type of cancer where too many immature granulocytes are getting produced (leukemic blasts) and as because they are not fully mature, they are not able to fight infections, as well as cause a shortage of red blood cells and platelets.

The maturation process of the bone cells occurs when osteogenic cells (the only ones which can divide) [differentiate](https://www.boundless.com/biology/definition/differentiate/) and turn into [osteoblasts](https://www.boundless.com/biology/definition/osteoblast/) (originally arising from mesenchymal stem cells) which are responsible for the formation of new bones. They synthesize a [collagen](https://www.boundless.com/biology/definition/collagen/) matrix and calcium salts and when the area around to the osteoblast calcifies, it becomes trapped and develops into an osteocyte which is the most mature type of the bone cell. Osteosarcoma, thereby, is caused by the disrupted differentiation where tumor cells are not able to differentiate into mature osteoblast cells which can form a proper bone.

Taking into account the issue of the cells maturation which leads to cancers, the approach for treatment in which malignant cells are forced to differentiate into mature forms applying chemical agents has emerged in the late 1980s (Murakami & Tashiro, 2015).

APL was the first hematological malignancy for which such therapeutic method was applied into clinical practice using all-trans-retinoic acid (ATRA). Soon, however, was discovered that ATRA was effective in APL with the translocation between 15 and 17 chromosomes, but not in other types of leukemia. The main purpose of such approach using ATRA was inducing cancer cells to get over the block of the differentiation and proceed to the apoptotic pathway instead of the killing cancer cells with cytotoxic therapies. That would help to avoid side effects and improve remission and cure rates. Induced differentiation of myeloid precursor cells should improve the immunity of patients avoiding massive lysis of white blood cells which can be seen in cytotoxic treatment (Gocek & Marcinkowska, 2011). The picture below demonstrates the principle of the differentiation approach using ATRA.



*Figure 2: PML-RAR-α transcriptional repression, RA-induced activation and differentiation therapy as a cure* (Ablain & The, 2011)

At the picture can be seen that the translocation between 15 and 17 chromosomes causes a merger of the promyelocytic leukemia genes and retinoic acid receptor alpha (RARα). This merger product forms homo-dimers, co-repressors and thus, suppresses expression of genes required for granulocytic differentiation (differentiation block). ATRA causes a conformation change of the molecule complex around PML-RARα. Thus, co-repessors are emitted, normal regulation of RARα genes is renewed and therefore, the differentiation of APL cells is stimulated (Koeffler, Nowak, & Stewart, 2009). Some studies, however, showed that treatments of APL with ATRA can give significant negative impact in the form of the relapse after remission in regard to 10%-30% patients, short remissions’ duration and retinoic acid syndrome.

In the case of AML, more than 200 chromosome translocations and mutations in leukemic cells exist and it is not know which of these mutations are responsible for expansion of abnormal cells and which of them are accumulated during disease. Taking into account this issue, it is obvious that finding of a particular treatment for AML is a challenging task (Gocek & Marcinkowska, 2011). Nowadays, a lot of scientific work is dedicated to identify other potent compounds which would be highly effective to force cancer cells to differentiate into their mature form without strong negative impacts, avoiding resistance developing and being universal in regard to cells with various mutations. This project is specifically aimed to test the effectiveness of some novel chemicals with regard to leukemia cell lines without t15:17 chromosome translocation, but with some other abnormalities. The chemicals which show the most effective results in leukemia cells differentiation will be tested on osteosarcoma cell line to check their effectiveness in regard to bone cancer in order to find out the chemical with wider cancer targeting.

## 2.4 Application of the MTT/NBT assays to leukemia cancer cell lines in vitro

MTT assay is an assay for measuring cellular metabolic activity and cytotoxicity where NAD(P)H-dependent cellular oxidoreductase enzymes under certain conditions represent the number of viable cells present. NAD(P)H-dependent cellular oxidoreductase enzymes are able to reduce the tetrazolium dye MTT to its insoluble [formazan](https://en.wikipedia.org/wiki/Formazan) and then dimethyl sulfoxide (DMSO) is added to dissolve it into a purple color solution. MTT assay requires incubation of the cells with a reagent in order to be able to transform a substrate to a colored solution which hereafter can be analyzed by the spectrophotometer where the generated signal is proportional to the amount of viable cells present.

Only viable cells which have active metabolism are able to convert MTT into a purple colored formazan which gives an absorbance of maximum 570 nm, therefore color formation acts as a marker of only the viable cells (Coussens, Nelson, & Sittampalam, 2004).

In contrast, NBT (nitroblue tetrazolium) assay is applied in order to define the production of superoxide anion (O2-) in cells. This assay is carried out by quantifying the cells having blue NBT formazan deposits, which are created by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium (Y-NBT) by O2- (Y., Choi, Kim, & Kim, 2006).

Therefore, this assay can detect the respiratory burst response of mature myeloid cells, since their differentiation is associated with the ability to generate a respiratory burst (release of superoxide radical) as they come into contact with [bacteria](https://en.wikipedia.org/wiki/Bacterium) or [fungi](https://en.wikipedia.org/wiki/Fungus) confirming their important role in immune system (Farzaneh, Freeman, Rayner, Trayner, & I., 1995).

Initially, NBT and MTT assays using different chemicals will be performed in regard to HL60 leukemia cell line and then subsequently to KG1a, K562 cell lines in order to identify the most effective ones referring to differentiation. That will be achieved by dividing NBT into MTT producing the value which would reflect the overall result regarding viability and maturation. HL60 and KG1A can maturate into granulocyte or monocyte, while K562 leukemia cell line can maturate only into granulocytes. Three concentrations of every chemical will be used, such as 0,1 µM, 1 µM and 10 µM. Also, three controls will be used along with the chemicals, such as DMSC, RPMI/IMDM/DMEM F12 and PMA100.

Moreover, the cytotoxicity test on healthy lymphocytes will be performed using MTT technique as well in order to confirm that chemicals applied for differentiation are not killing the healthy blood cells.

Regarding osteosarcoma SaOS2 cell line, MTT with relation to cytotoxicity and the alkaline phosphatase (ALKP) tests described in the next subchapter will be carried out.

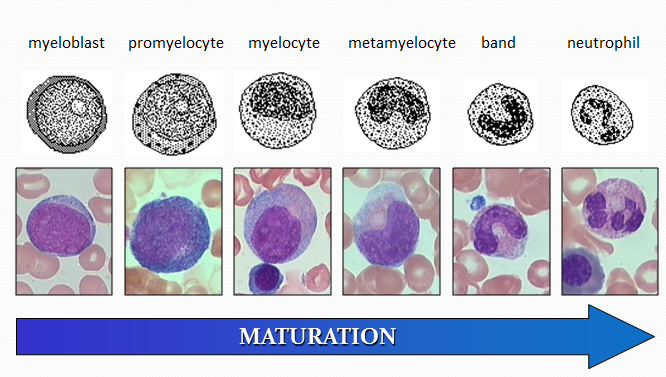
## 2.5 Alkaline phosphatase assay

Alkaline phosphatase enzyme (ALP) is a biochemical marker for the activity of osteoblasts which plays a role in the mineralization of bones. Adding the lysis solution and then freeze-thawing the cells twice at -20 degrees helps to break cellular membranes and extracts enzymes to the environment. P-nitrophenyl phosphate (PNPP) which is a non-proteinic and non-specific substrate used in this assay triggers the enzyme activities. Phosphatases have the ability to catalyze the hydrolysis of PNPP to *p*-nitrophenol, a chromogenic product with absorbance at 405 nm (MacKintosh, 1993). Thus, the activity of ALP can be measured spectrophotometrically in a micro titer plate format.

## 2.6 Cancer cells morphology analysis

Morphology analysis by a microscope in culture has to be carried out after differentiation procedure in order to evaluate visually the effect of chemicals in regard to the level of cells maturation (differentiated, partial or undifferentiated) and viability. Viability is counted taking into account a percentage ratio of apoptotic cells and live cells after the treatment, while maturation level has to take into consideration such characteristics as the amount of nucleoli, the nuclear-cytoplasmic ratio (ratio of the nucleus volume compared to the cytoplasm size of the cell), the presence or the absence of euchromatin (form of chromatin with DNA, RNA and protein which is lightly packed) and heterochromatin (genetic material of different density from normal where the genetic is changed or suppressed), the amount of metaphases (second stage of the cellular division), the amount of cells with lobed nuclei, the density of the chromatin. It is worth noting that the presence of euchromatin and heterochromatin provides the information about the cell activity. Euchromatin is prevalent in cells which are active in the transcription of their genes while heterochromatin is prevalent in cells that are less active or with the absence of activity (Allis & Jenuwein, 2016).

The positive differentiation would be obtained if the high percentage of the cells became more mature along with the high level of cellular viability. In order to avoid being bias, the table in Excel for the calculating the percentages will be used to find the scores and then to identify whether the cells are differentiated, partially differentiated or without differentiation. The picture below shows the maturation process of myeloid cells proceeding from its immature stage on the left to its mature stage on the right.

****

*Figure 3: Myeloid cells maturation process*

It can be observed that as the cell is getting more mature, the amount of cytoplasm in regard to the rest of the cell increases. The immature cell’s nucleus is round and is proportionally big in regard to the rest of the cell. However, as the cell is getting more mature, the nucleus decreases in size and can take different shapes.

In immature stage of the cell the chromatin develops from a fine and delicate structure into more coarse and clumped. In addition, in myelogenous cells, granules emerge in the cytoplasm as the cell matures. In the beginning these granules are few, but as the maturation continues further, the granules grow in number.

Before performing morphological analysis of cells, cytocentrifuge technique with subsequent Leishman’s staining should be performed. In addition, trypsinization applying trypsin which breaks down the protein has to be executed particularly for osteosarcoma cell line in order to dissociate cells attached to the flask after their culturing.

Cytocentrifuge is a technique which concentrates the cells on a slide in even monolayer 6 mm in diameter to enhance the morphological characteristics of the cells. It is required a fresh sample with the cells of a suitable concentration, the lack of interfering materials and a suspension fluid for a proper cell precipitation. It is important to adjust the right concentration of the cells since if too high amount of cells is used, the specimen will be thick, multi layered and hard to read (Stokes & Logan, 2004).

After the cells have been prepared on the slide, Leishman’s staining should follow. It is based on a methanolic solution of methylene blue and eosin. Eosin is a negatively charged acidic dye, so it stains positively charged particles, such as granules of eosinoplhils, while methylene blue is a positively charged basic dye which stains negatively charged particles such as granules of basophils, cytoplasms and nuclei of white blood cells. Methyl alcohol performs the function of the fixative, so the cells due to the precipitation of the proteins are getting fixed to the slide and cannot be washed out. In general, it is able to make clear distinction between staining shades and stain granules differentially, therefore is widely used in [microscopy](https://en.wikipedia.org/wiki/Microscopy) in order to stain [blood and bone marrow smears](file:///C:\Users\user\AppData\Roaming\Microsoft\Word\blood%20and%20bone%20marrow%20smears) (Kaler & Shameem, 2016).

# METHODS

In order to answer the research question different sets of the experiments have been performed. The description of all subsequent steps is presented below:

# 

## 3.1 Cell lines culturing

***Culture media preparation***

IMDM and DMEM F12 media suitable for culturing KG1A and osteosarcoma cells respectively were prepared in the same way by adding 11.70 g of IMDM or DMEM F12 powder (PAN Biotech, Germany) in 1 liter of distilled water. The flask was placed on the stirrer for 15-20 minutes to dissolve the powder. Then, 1.2 g of NaHCO3 (Sigma-Aldrich, United States) was added to the flask to adjust the right pH. The prepared solution was transferred to the filtering kettle where by means of the applied pressure and 40-micron filter it was purified. Then, the obtained solution was transferred to 5 flasks and 200 µl of penicillin/streptomycin solution along with 25 ml of the fetal bovine serum (FBS) were added to every flask. RPMI media suitable for culturing Hl60 and K562 leukemia cells, was taken from the sterile stock (Gibco, United States) with subsequent adding of the same amount of penicillin/streptomycin and FBS.

***Thawing procedure***

The thawing procedure was started by removing the cryovial with the frozen cells (approx. 1 ml) from -80ᵒC. The cryovial immediately was placed into a 37ᵒC water bath for no longer than 1 minute until the moment when there is just a little bit of ice left. Then, the vial was transferred to a safety cabinet and before opening it was wiped with 70% ethanol. 6 ml of pre-warmed RPMI, IMDM or DMEM F12 culture medium was transferred slowly drop-wise into larger centrifuge tube which contained the thawed cells. The cell suspension was centrifuged at 2000 rpm for 5 minutes. After completing the centrifugation, the clearness of the supernatant and visibility of the pellet were checked. Then, the supernatant was aseptically decanted avoiding disturbing the pellet. Afterwards the pellet was re-suspended in 6 ml of suitable medium and then again centrifuged at 2000 rpm for 5 minutes. Afterwards, the supernatant was discarded and the cells were re-suspended in 5 ml of growth medium with their subsequent transferring into the suitable culture vessel (T75) containing 7 ml of suitable culture medium.

The cell suspension was incubated for 24/48 hours at 37ᵒC in the humid incubator containing 5 % of CO2. Cells were checked by the microscope daily in order to ensure that they are growing and that they look round or elongated in shape, plump and refracting light around their membranes.

***Sub-culturing for suspension cell lines***

Sub-culturing was performed when cells had reached confluence and started overgrowing.

If cells were 70-80% confluent (covered 70% of flask surface), they were splitted in 1:2 to make them ready for the experiments in 1-2 days. If less than 70-80% confluent, but the experiment will be performed only after 3-4 days, then they were splitted at a lower split ratio 1:5. To perform sub-culturing, the culture medium was warmed in incubator at 37ᵒC for minimum 30 minutes. The required volume of cell suspension was taken from the flask using sterile pipette and transferred to the new flask. For 1:2 split from 12 ml of the suspension 6 ml was taken. Then, 6 ml of pre-warmed media was added to cell suspension. Afterwards, flasks were immediately placed into 37°C CO2 incubator.

***Sub-culturing for attached cell lines***

PBS solution was prepared in the proportion of 9.6 g in one liter. All the media was carefully removed from the flask avoiding any contamination risks. Then, cells were washed two times with 5 ml of PBS solution using sterile pipette. Then, 5 ml of trypsin was added to the flask leaving it for 5 minutes at the room temperature or 2 minutes in the incubator. The flask was tapped from all the sides to make sure that cells are detached from the surface. At this point trypsin has to look cloudy or turbid, so it has to be discarded with subsequent placing the flask to the tube containing 5 ml of fresh media (1:1). Afterwards, the suspension was centrifuged for 5 minutes at 1500 rpm. Then, the supernatant was discarded and fresh media was added to re-suspend the cells. The cells were transferred into new flask T75 and then 7 ml of fresh media was toped up to give volume of 12 ml in total. Afterwards the flask was kept for 24/48 hours at 37ᵒC with 5% CO2 in the incubator.

***Cells counting and concentration adjustments***

The glass cover slip of the hemocytometer was cleaned with 70% ethanol and placed over the counting chamber. One drop of the cellular suspension was loaded between the glass cover slip and the chamber. By placing the chamber under Moticam Pro (AE2000) microscope the cells were counted from 4 (4x4) squares and the average value of cells in one milliliter was estimated. The adjustments were made taking into account the final required volume and the actual average cell concentration in one milliliter to adjust it in a way that the concentration would correspond to 2x105 cells/ml in a required final volume.

## 3.2 NBT/MTT assays

The following solutions had been prepared sterilely beforehand: 20 µM, 2 µM and 0.2 µM in RPMI, IMDM or DMEM F12 (depending on the cell type used) for every chemical to test, 1.6 % DMSO, 100 nM PMA, 1M HCL, 2M KOH, 5 mg/ml -1 MTT/PBS solution, 0.2 % w/v NBT/PBS. MTT/PBS and NBT/PBS solutions were kept closed and airtight as much as possible in order to prevent oxidation. Afterwards, the seeding of the plates was proceeded. All 4 plates (2 for day 3 and 2 for day 5 if testing 4 chemicals at the same time) were labelled and 50 µl of the cellular suspension with the concentration of 2x105 cells/ml was pipetted in every well in a sterile way. Then, 50 µl of the prepared chemical dilutions was pipetted, followed by 50 µl controls pipetting and sterile PBS to surrounding wells. Afterwards, the plates were placed in incubator for day 3 and day 5. The positive controls DMSO 1.6 % and PMA100 were used for granulocytic differentiation and monocytic differentiation respectively, while RPMI, IMDM or DMEM F12 as a negative control. After the seeding and incubation of the plates, the NBT/MTT procedures were performed in a following way:

* ***Beginning of MTT***

Cells were checked and the observations recorded. Then, the plates were shaken at 750 rpm for 5 minutes. 20 µl of 5 mg/ml-1 MTT/PBS was added to each well in the original well and incubated for 4 hours.

* ***Performing NBT***

The other new plate was centrifuged at 2000 rpm for 10 minutes, then, the supernatant was discarded. While plates are centrifuging, PMA was added to NBT for activation in proportions of 1:100 (100 µl PMA in 10 ml NBT). After, the medium was completely removed by turning plate on a tissue. Wells were filled with 50 µl activated NBT and incubated at 37ᵒC for 20 minutes. Then, 70 µl of 1 M HCl was added to stop NBT reaction and then subsequently centrifuged at 2000 rpm for 10 minutes. After, the supernatant was discarded and 50 µl 2 M KOH and 150 µl of DMSO was added. Then, the absorbance was measured at 630 nm with 405 nm reference wavelength.

* ***Continuation of MTT***

After 4 hours of incubation, the plates were centrifuged at 2000 rpm for 10 minutes, with subsequent removal of medium by turning plate on a tissue. 120 µl of DMSO was added to each well and the plate was placed on a plate shaker for 5 minutes. Then, the absorbance was measured at 562 nm with 570 nm as the reference wavelength.

## 3.3 Cytotoxicity test on lymphocytes

CPD solution was prepared by mixing 26.3 g sodium citrate (dihydrate), 25.5 g dextrose (monohydrate), 3.27 g citric acid, 2.22 g monobasic sodium phosphate (monohydrate) and toped up to 1 liter with distilled water.

ELB was prepared by mixing 0.04 g EDTA, 7.49 g ammonium chloride, 0.745 potassium chloride, 0.79 g ammonium carbonate up to 1 liter with distilled water.

The test tube with 7mL CPD was labelled and approx. 25 ml of collected blood was immediately added to the CPD tube. Then, the tube was filled with PBS up to 50 ml as soon as possible in order to prevent blood from coagulation.

Then, a new tube was labelled and 10mL histopaque was prepared in it. The blood:PBS sample was cautiously and dropwise added on top of the histopaque layer without disturbing it with subsequent centrifugation at 2500 rpm for 25 minutes. The plasma was removed and the white hazy layer was collected into a new tube. Red blood cells layer was avoided as much as possible. 3:1 ELB:PBS solution was added to the sample, mixed, incubated for 10 minutes and shaken at intervals. Then, it was centrifuged at 1500rpm x 5 minutes. The step with adding 3:1 ELB:PBS solution and centrifugation was repeated several times until a white pellet was collected. Next, the supernatant was removed and 5mL media was added to sample. It was left for overnight for the monocytes to adhere. Then, the lymphocytes (the floating cells) were collected in a new T25 flask at a concentration of 2-3 × 105 cells  mL-1 in RPMI. Afterwards, phytohaemagglutinin was added at a concentration of 2% v/v for activation and the cells were left for 48 hours before being seeded at 1 × 105 cells per well in 96-well plates. 50 µl of the cellular suspension and chemicals were used for seeding. Then, MTT assay was performed at first, second and third days.

## 3.4 Centrifugation and Leishman’s staining

Centrifugation was performed by using 200 µl of cellular suspension with the concentration of 1x105 cells/ml and subsequent centrifugation of the smear slide at 1000 rpm for 5 minutes. Leishman’s stain solution was prepared by mixing and dissolving 0.15 g of Eosin-Methylene blue in 100 ml of Methanol and left for dissolving overnight. The container then was covered with Parafilm in order to prevent contamination by moisture. Then, the solution was filtered by using a dry Whatman paper filter in a clean and dry brown glass bottle. The solution was aged 3 days before using it for the first time and stored in tightly sealed bottle protecting from light and heat and far from the bottles containing acid. Then, the smears which are as thin as possible and air-dried were fully covered with Leishman’s stain solution for 2 minutes. Twice of the amount of PBS was added and mixed by swirling with subsequent incubating for at least 10 minutes. Then, the smears were rinsed thoroughly with tap water and air dried.

## 3.5 Morphology analysis

Morphology analysis of Hl60, K562 and KG1a cell lines was performed by visual assessment of the morphological characteristics (listed on the left column of the table below) in respect that certain percentage range of the cellular population corresponds to particular score. The scoring of every smear was performed under three High Powered Fields x4, x10, x40 (abbreviated in the table as HPF1, HPF2, HPF3). The scores are observed on the last three columns and normally appear only after choosing the percentage of cells (selection function visible only in Excel program). Then, the scores from every HPF were summed up in order to obtain a total average number and conclude whether the cells have been differentiated, partially differentiated or undifferentiated taking into account that 161 is the maximum score and 1 is the minimum. Also, the percentage of apoptotic and live cells was found using three High Powered Fields. The total average scores and the percentages of the dead and live cells were compared with each other to find out the most efficient chemical used for differentiation.

*Table 2: Leukemia morphology appearance for differentiation status assessment*



## 3.6 Alkaline phosphatase assay

Alkaline phosphatase activity of the cells after the treatment with the chemicals was determined biochemically at the third and the fifth days. The cells were rinsed twice with Ringer solution after the incubation period. Then, 250 µl of lysis solution containing 10 mM Tris HCl pH 7.5, 0.5 mM MgCl2 and 0.1% Triton X-100 was added to every well and then mixed for 5 minutes on the shaker. The cellular material was homogenized by two freeze-and-thaw cycles. Afterwards 50 µl of the cellular suspension was transferred from every well to a new plate with subsequent adding 50 µl of p-nitrophenyl phosphate (4.34 mM) in 100 mM glycine, pH 10.3, 1mM MgCl2 and incubation at 37ᵒC for 30 minutes. Enzyme activity was quantified by absorbance measurements at 405 nm.

# RESULTS

The following chapter contains the results of the experiments made during this project.

## 4.1 NBT/MTT assay on leukemia cells

This subchapter represents NBT/MTT screen results with relation to leukemia cell lines, such as Hl60, K562, KG1A. The subsections below highlight the obtained results individually for every leukemia cell type.

## NBT/MTT assay on Hl60 cell line

Initially NBT/MTT screen was performed using anonymous chemicals such as Dp-132, Dp-133, Dp-137, mag-218, mag-221, mag-234, mag-243, mag-244, mag-261, NV-139, NV-140, Il-10, Il-11, Il-12, VC-82, VC-85, Fotis, CLB, 550, 551, 553, 554, 555, 556, 559, 560 on Hl60 cell line. Every chemical has been seeded with the concentrations 10 µM, 1 µM and 0,1 µM in four replicates for every concentration. The diagrams shown in this subsection contain the most favorable chemicals with its particular concentration obtained during NBT/MTT screen regarding HL60 cell line. Such chemicals are emphasized by gradient color.

*Figure 4: NBT/MTT screen histogram performed on the third day using HL60 cell line. The chemicals Dp-137, mag-261, Fotis, Dp-133 with the concentrations 10 µM, 1 µM, 0.1 µM are located on x-axis with the absorbance on y-axis. RPMI was used as a negative control, DMSO and PMA as positive controls for granulocytic and monocytic differentiation respectively*

*Figure 5: NBT/MTT screen histogram performed on the third day using HL60 cell line. The chemicals mag-244, mag-218, IL-12, Dp-132 with the concentrations 10 µM, 1 µM, 0.1 µM are located on x-axis with the absorbance on y-axis*

The chemicals Dp-137 and Dp-132 with the concentrations 10 µM have shown favorable results in NBT/MTT screen on the third day demonstrated in two diagrams above. Their absorbance values are above values of the control RPMI and DMSO control indicating potential granulocytic differentiation. MTT diagrams reflecting cytotoxicity do not show too low absorbance for Dp-132 and Dp-137 (0,525 and 0,457 respectively) which indicates that presumably they are not cytotoxic for Hl60.

The next two diagrams represent the chemical VC-82 which showed a good result in NBT/MTT screen for both Day 3 and Day 5 since its values are above the controls. However, a separate MTT diagram showed lower values for VC-82 than for previously mentioned chemicals and are 0,110 on the third day and 0,126 on the fifth day.

*Figure 6: NBT/MTT screen histogram performed on the third day using HL60 cell line. The chemicals IL-10, IL-12, VC-82, VC-85 with the concentrations 10 µM, 1 µM, 0.1 µM are located on x-axis with the absorbance on y-axis*

*Figure 7: NBT/MTT screen histogram performed on the fifth day using HL60 cell line. The chemicals IL-10, IL-12, VC-82, VC-85 with the concentrations 10 µM, 1 µM, 0.1 µM are located on x-axis with the absorbance on y-axis*

It can be observed that in two NBT/MTT diagrams below the chemical NV-139 with the concentration 10 µM and 1 µM demonstrates good results for both day 3 and day 5. The chemical NV-140 with the concentration 10 µM shows a favorable result only on day 3. However, MTT values of these chemicals are quite low: 0,095 for NV-139 (10µM), 0,085 for NV-139 (1 µM), 0,083 for NV-140 (10 µM) on the third day and 0,106 for NV-139 (10 µM), 0,103 for NV-139 (1 µM) on the fifth day.

*Figure 8: NBT/MTT screen histogram performed on the third day using HL60 cell line. The chemicals NV-139 and NV-140 with the concentrations 10 µM, 1 µM, 0.1 µM are located on x-axis with the absorbance on y-axis*

*Figure 9: NBT/MTT screen histogram performed on the fifth day using HL60 cell line. The chemicals NV-139 and NV-140 with the concentrations 10 µM, 1 µM, 0.1 µM are located on x-axis with the absorbance on y-axis*

## NBT/MTT assay on K562 cell line

The most efficient chemicals which have been found in NBT/MTT screen with relation to HL60 cell line were further tested on K562 cell line with the exception of Dp-132 since it has been lost. The NBT/MTT diagram below shows that all the chemicals have an effect on K562 cell line as well. Dp-137 shows slightly lower values, but still higher than DMSO control.

*Figure 10: NBT/MTT screen histogram performed on the third day using K562 cell line. The chemicals Dp-137, VC-82, NV-140 with the concentration 10 µM, and NV-139 with the concentrations 10 µM and 1 µM are located on x-axis with the absorbance on y-axis*

Nonetheless, MTT test showed that MTT absorbance values of the chemicals NV-140 (10 µM) and NV-139 (10 µM) for both day 3 and day 5 are quite low and are 0,091 and 0,092 respectively which might indicate that these chemicals are too cytotoxic for K562. The NBT/MTT results on the fifth day represented in the diagram below still showed an effect of the chemicals, but with lower values for Dp-137 (10 µM) and NV-139 (1 µM).

*Figure 11: NBT/MTT screen histogram performed on the fifth day using K562 cell line. The chemicals Dp-137, VC-82, NV-140 with the concentration 10 µM, and NV-139 with the concentrations 10 µM and 1 µM are located on x-axis with the absorbance on y-axis*

## NBT/MTT assay on KG1A cell line

Dp-137, VC-82, NV-140 and NV-139 had been tested on KG1A cell line as well and the results on the third day are represented in the diagram below. All chemicals in NBT/MTT test on the third day show high values above DMSO and PMA controls along with all MTT values above 0,100.

*Figure 12: NBT/MTT screen histogram performed on the third day using KG1A cell line. The chemicals Dp-137, VC-82, NV-140 with the concentration 10 µM, and NV-139 with the concentrations 10 µM and 1 µM are located on x-axis with the absorbance on y-axis*

The next diagram below shows the results which were obtained on the fifth day with all the values above PMA, but lower than DMSO. The MTT values of NV-140 (10 µM) and NV-139 (10 µM) are 0,092 for both.

*Figure 13: NBT/MTT screen histogram performed on the fifth day using KG1A cell line. The chemicals Dp-137, VC-82, NV-140 with the concentration 10 µM, and NV-139 with the concentrations 10 µM and 1 µM are located on x-axis with the absorbance on y-axis*

The chemicals Dp-137 (10µM), VC-82 (10µM), NV-140 (10 µM) and NV-139 (1 µM and 10µM) were included in morphology assay in order to confirm visually their effect on the cells and evaluate whether low MTT values are caused by their cytotoxic effect.

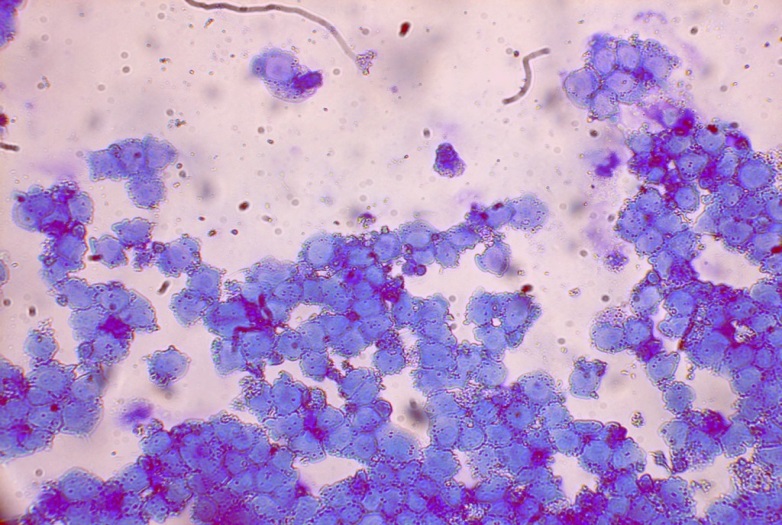
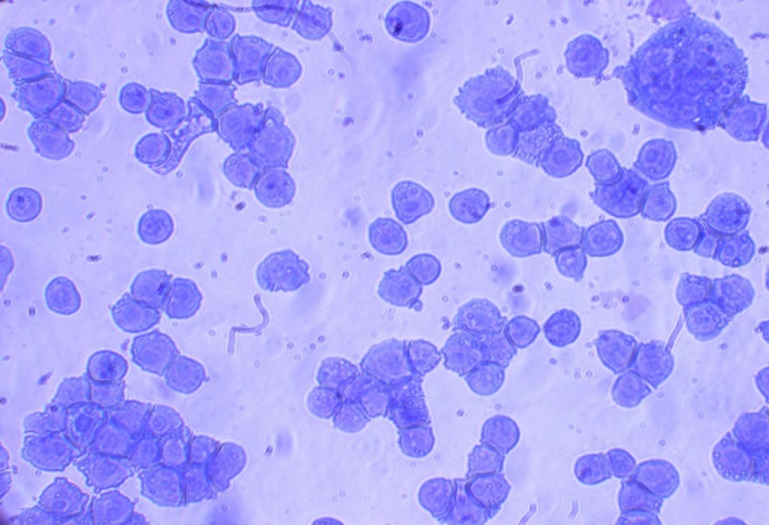
## 4.2 Morphological analysis of leukemia cell lines

## 4.2.1 Morphological assessment of HL60 cell line

*Table 3: Morphology results for HL60 cell line representing the scores at the third and the fifth days, the corresponding percentage of dead cells and MTT values*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Name of chemicals*** | ***Scores Day3 (Avg)*** | ***% Dead cells***  ***(Avg)*** | ***MTT values*** | ***Scores Day5 (Avg)*** | ***%Dead cells***  ***(Avg)*** | ***MTT values*** |
| Dp-137 (10µM) | 83,33 | ≤ 10% | 0,526 |  |  |  |
| Dp-132 (10µM) | 63,00 | < 10% | 0,457 |  |  |  |
| VC-82 (10µM) | 36,33 | ≈50% | 0,110 | Cytotoxic | > 90% | 0,126 |
| NV-140 (10µM) | Cytotoxic | > 90% | 0,083 |  |  |  |
| NV-139 (10µM) | Cytotoxic | ≈90% | 0,095 | Cytotoxic | > 90% | 0,106 |
| NV-139 (1µM) | Cytotoxic | ≈90% | 0,085 | Cytotoxic | ≈90% | 0,103 |

The results of morphology regarding HL60 cell line represented in the table above showed that such chemicals as NV-139 (10µM, 1µM), as well as VC-82 (10 µM) were too cytotoxic at both the third and the fifth days. In addition, NV-140 (10 µM) was too cytotoxic at the third day as well. The scoring table was not possible to apply in these cases since the cells have been significantly degraded and lost any characteristics of the normal cellular morphology. The grey rows in a table above indicate a non-completion of the morphology assay, since NBT/MTT test did not show results which would indicate a presumable cellular differentiation. The most successful results were achieved applying Dp-137 chemical (10 µM) which gave a score of 83,33 indicating a partial cellular differentiation. Moreover, the percentage of the dead cells is not higher than 10% which is not too high. Slightly less efficient results were achieved with Dp-132 which also gave a partial differentiation with approximately 10% of dead cells. VC-82 corresponds to quite low score of differentiation with quite high percentage of dead cells. It is worth noting that the cytotoxicity effects observed in the morphology assay coincide with low MTT values found in the previous experiments. The pictures below represent the most successful morphology result applying Dp-137 regarding HL60 compared with the control RPMI where untreated HL60 cells are shown. In the right picture quite high population of cells which obtained granulas and vacuoli, irregular cytoplasm boarders, heterochromatin and some other characteristics peculiar to differentiated cells, such as the decrease of the amount of nucleoli were clearly observed.



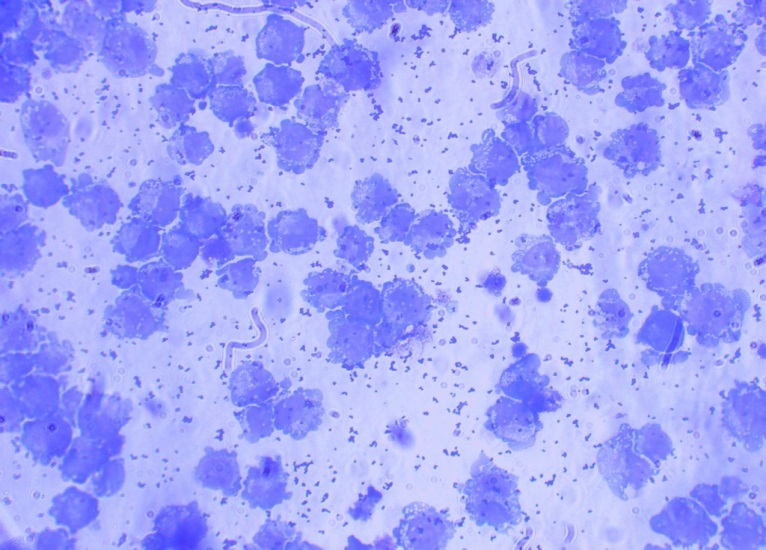
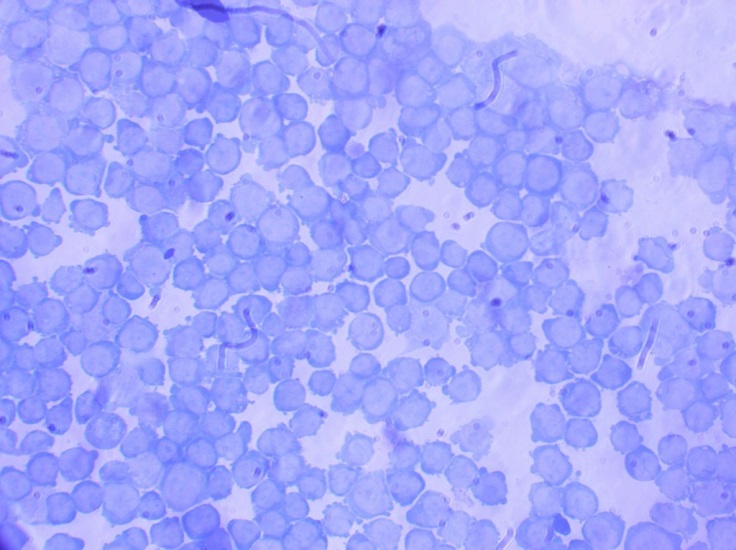
*Figure 14: The morphology result representing the partial differentiation of HL60 cell line at the third day applying Dp-137 with the concentration of 10 µM (on the right) compared with the control RPMI (on the left) at the magnification x40*

## 4.2.2 Morphological assessment of K562 cell line

*Table 4: Morphology results for K562 cell line representing the scores at the third and the fifth days, the corresponding percentage of dead cells and MTT values*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Name of chemicals*** | ***Scores Day3 (Avg)*** | ***% Dead cells***  ***(Avg)*** | ***MTT values*** | ***Scores Day5 (Avg)*** | ***%Dead cells***  ***(Avg)*** | ***MTT values*** |
| Dp-137 (10µM) | 109,00 | < 10% | 0,366 | 102,66 | < 10% | 0,577 |
| VC-82 (10µM) | 61,00 | ≈ 30% | 0,176 | 57,00 | ≈ 35% | 0,126 |
| NV-140 (10µM) | Cytotoxic | ≈ 80% | 0,091 | Cytotoxic | ≈ 80% | 0,091 |
| NV-139 (10µM) | Cytotoxic | ≈ 70% | 0,092 | Cytotoxic | ≈ 70% | 0,092 |
| NV-139 (1µM) | 62,00 | < 10% | 0,207 | 65,00 | < 10% | 0,643 |

The results of morphology regarding K562 cell line presented in the table above show that Dp-137 (10µM) is also effective towards K562 cell line since it gave a score of 109,00 at the third and 102,66 at the fifth days along with quite low percentage of dead cells. The treated cells showed even more expressed morphology of differentiated cells than in the case with HL60 cell line due to the bigger amount of granulas and cells with highly irregular cytoplasmic boarders. NV-139 (1 µM) showed a slight differentiation of certain cells, however it is not too significant. In the case of VC-82 (10µM) approximately 30% of cells population was dead which means that the chemical is quite cytotoxic for that type of leukemia cell line.



*Figure 15:* *The morphology result representing the partial differentiation of K562 cell line at the third day applying Dp-137 with the concentration of 10 µM (on the right) compared with the control RPMI (on the left) at the magnification x40*

## 4.2.3 Morphological assessment of KG1a cell line

*Table 5: Morphology results for KG1A cell line representing the scores at the third and the fifth days, the corresponding percentage of dead cells and MTT values*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Name of chemicals*** | ***Scores Day3 (Avg)*** | ***% Dead cells***  ***(Avg)*** | ***MTT values*** | ***Scores Day5 (Avg)*** | ***%Dead cells***  ***(Avg)*** | ***MTT values*** |
| Dp-137 (10µM) | 39,00 | ≈ 50 % | 0,122 | 42,00 | > 60% | 0,142 |
| VC-82 (10µM) | 47,00 | ≈ 30 % | 0,137 | Cytotoxic | > 90% | 0,134 |
| NV-140 (10µM) | Cytotoxic | > 90% | 0,102 | Cytotoxic | > 90% | 0,092 |
| NV-139 (10µM) | Cytotoxic | > 90% | 0,103 | Cytotoxic | > 90% | 0,092 |
| NV-139 (1µM) | Cytotoxic | > 90% | 0,104 | Cytotoxic | > 90% | 0,109 |

In the case of KG1A Dp-137 (10 µM) was not effective for the differentiation, because only a few cells showed some slightly positive results regarding differentiation and it also caused a high amount of dead cells. Moreover, the chemicals NV-140, NV-139, VC-82 were too cytotoxic for that type of leukemia cell line which is also reflected in quite low values of MTT.

## 4.3 Cytotoxicity test using lymphocytes

This subchapter demonstrates the results obtained performing cytotoxicity test with extracted healthy lymphocytes applying the most efficient chemicals found in NBT/MTT screen for Hl60 leukemia cell line. The experiment was performed during three days with the seeding of the plates at Day 0 and subsequent incubation of the plates for overnight. RPMI was used as a negative control. The chemicals Dp-137 (10µM), VC-82 (10µM), NV-140 (10µM), NV-139 (10µM and 1µM) have been used for seeding along with cellular suspension and formed violet product as a marker for cellular activity was measured spectrophotometrically.

*Figure 16: The results of cytotoxicity MTT test on lymphocytes*

The obtained results showed that the chemicals do not have cytotoxic effect on the healthy lymphocytes at the first, the second and the third days. It can be seen that at the third day there is a rapid decline in absorbance compared with the first and the second days, however, it is not significantly lower than its corresponding control which indicates its non-cytotoxicity. Such rapid decline in absorbance at the third day might be due to the fact that lymphocytes were grown outside the human body and thereby naturally lost some of their cellular activity.

## 4.4 Cytotoxicity test using osteosarcoma

The diagram below shows that such chemicals as DP-137 (10 µM), VC-82 (10 µM), NV-140 (10 µM) are not cytotoxic regarding SaOS2 cell line at the third and fifth days, while NV-139 (10 µM and 1 µM) was significantly cytotoxic.

*Figure 27: The results of cytotoxicity MTT test on osteosarcoma*

## 4.5 Alkaline phosphatase assay

Alkaline phosphatase assay performed on SaOS2 cell line on the third day did not produce any reliable values which could be taken into account since they were too low and showed a big correlation between each other. It might be due to the fact that not all of the cells in the wells have been equally lysed or the amount of the produced enzyme was too small. The diagram presented below reflects the results obtained at the fifth day which show that no any of the chemicals used in the test could lead to increased activity of the alkaline phosphatase enzyme. All the absorbance values corresponding to the chemicals did not exceed the value of the negative control DMEM F12.

*Figure 18: Alkaline phosphatase level after the differentiation in SaOS2 cell line at the fifth day*

# DISCUSSION

Initially NBT/MTT assay was performed on HL60 using 26 chemicals from different screens in order to identify the most efficient ones which would be selected for further tests with K562 and KG1A. It was found out that only such chemicals as Dp-137 (10µM), Dp-132 (10µM), VC-82 (10µM), NV-140 (10µM), NV-139 (10µM; 1 µM) show the most favorable results in NBT/MTT test. Dp-140, Dp-132 and Dp-137 show a favorable result only at the third day, while others at the third and the fifth days. However, all the chemicals with the exception of Dp-137 and Dp-132 showed quite low values in a separate MTT test which presumably could indicate their cytotoxicity in regard to HL60. The morphological test confirmed that Dp-132 and particularly Dp-137 are the most efficient chemicals for HL60 which caused a partial differentiation with the amount of dead cells not higher than 10%, while others showed quite high cytotoxicity.

Subsequent NBT/MTT tests showed that all the chemicals tested on HL60 have a positive effect on K562 and KG1A cell lines as well. Nevertheless, some of the chemicals tested on these cell lines corresponded to quite low MTT values which were lower or slightly higher than 0,100. The morphological test confirmed that low MTT values indicate high chemical cytotoxicity. It was found out that Dp-137 (10 µM) showed even better result on K562 cell line than with the case of HL60 which leaded to better cellular differentiation at the third and the fifth days. Such chemicals as VC-82 (10 µM) and NV-139 (1µM) showed a slight differentiation of certain K562 cells at the third and the fifth days along with quite high cytotoxicity. Due to the loss of Dp-132 the further tests on K562 and KG1A cell lines have not been executed.

Regarding KG1A such chemicals as NV-140 (10µM), NV-139 (10µM), NV-139 (1µM) showed the amount of dead cells higher than 90% for both the third and the fifth days. The chemicals Dp-137 (the third and the fifth days) and VC-82 (the third day) showed a slight differentiation of certain cells along with quite high percentage of apoptotic cells.

Subsequent MTT tests on cytotoxicity revealed that all above mentioned chemicals do not have any cytotoxicity effect on healthy lymphocytes.

The last set of the experiments devoted to osteosarcoma showed, however, that Dp-139 with the concentrations of 1 µM and 10 µM are too cytotoxic for SaOS2 cell line. All the chemicals used for the differentiation of leukemia cell lines, such as Dp-137, VC-82, NV-140, NV-139 did not increase the production of alkaline phosphatase enzyme at the third and the fifth days which most likely implies that they do not trigger the differentiation of SaOS2 cell line.

# CONCLUSIONS AND RECOMMENDATIONS

Dp-137 is the chemical which would be potentially interesting for further cancer research since it showed quite good results regarding cellular differentiation with respect to HL60 and K562 cell lines without too low levels of viability. Also, MTT cytotoxicity test showed that it is not cytotoxic for healthy blood cells, such as lymphocytes. That presumably means that Dp-137 does not have such harmful effect as chemicals used in chemotherapy. In order to confirm the obtained results regarding Dp-137 it is essentially to perform flow cytometry analysis which would help to identify the type of the leukemia cells after their differentiation. In the course of my project I did not manage to perform any tests with flow cytometry due to the lack of time and specialized skills working with flow cytometry equipment. Unfortunately, Dp-137 did not show significantly positive results regarding KG1A cell line as in the case with HL60 and K562, since that type of leukemia cells was quite challenging to force to differentiate and in most cases the application of the chemicals caused a death of the cells.

The experiments with lymphocytes showed that all the chemicals used in morphology test are not cytotoxic which means that they can be still potentially interesting in the experiments with other types of leukemia cell lines.

In general, it is essentially to continue screening the chemicals applying NBT/MTT, morphology and flow cytometry assays regarding leukemia cells to identify other potentially effective substances which would be effective for osteosarcoma as well.

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