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„Substituted thiosemicarbazones as effective inhibitors of leukemic cell growth “

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Abbreviation Index

ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATP	adenosine triphosphate
Ara-C	arabinofuranosylcytosine
ATCC	American type culture collection
ATRA	all-trans-retinoic acid
ATO	arsenic trioxide
Bax	Bcl-2-associated X protein
Bak	Bcl-2-homologous antagonist/killer protein
Bcl-2	B-cell lymphoma 2
CDK	cyclin-dependant kinases
CDR	deoxycytidin kinase
Chk1	checkpoint kinase 1
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CR	complete remission
dATP	deoxyadenosine triphosphate
dFdC	Gemcitabine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FLT3	FMS-like tyrosine kinase receptor 3
FMS	Feline McDonough Sarcoma
G0, G1, G2	gap-, or growth phases of the cell cycle
H3PO4	ortho-phosphoric acid
HL-60	acute promyelocytic leukemia cell line
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HU	hydroxyurea
IC50	half maximal inhibitory concentration
IL-1α	interleukin 1-alpha
M	molar
mM	millimolar
M-phase	mitosis
NaCl	sodium chloride
p21	cyclin-dependent kinase inhibitor 1
p53	tumor suppressor protein; transcription factor
p53R2	homolog of R2
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PBS/T	phosphate buffered saline/ tween
pH	pondus hydrogenii
PI	propidium iodide
PIC	protease inhibitor cocktail
PML	promyelocytic leukemia
PMSF	phenylmethylsulfonylfluoride
PVDF	membranes for western transfer and sequencing
QSAR	quantitative structure-activity relationship
R1	large α2-homodimer of ribonucleotide reductase
R2	small β2-homodimer of ribonucleotide reductase
RAR-α	retinoic acid receptor alpha
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	cell culture medium
SDS	sodium dodecyl sulphate
RR	ribonucleotide reductase
SCT	stem cell transplantation
S-phase	DNA-synthesis
TBS	tris buffered saline
TBS/T	tris buffered saline/tween
TCA	trichloroacetic acid
Tf	iron transport protein transferrin
TfR1	transferrin receptor 1
TNF	tumor necrosis factor
TNO	Tri-n-octylamine

1. Project Goals

Cancer is generally understood to be a genetic disease in which certain cells in the human (or animal) body divide continuously and spread into the surrounding tissues. In a healthy person cells grow and divide to form new cells as they constantly replenish dying cells in the body. Growth patterns of healthy cells are highly regulated by several inhibitors. Cancer cells, however, are able to grow uncontrolled by the host organism and become invasive.

To this day, more than 100 different types of cancer are known [2]. It is the second leading cause of death worldwide. A multitude of treatments has been developed thus far. Recent efforts focus on finding and developing new antineoplastic agents.
Though several cytostatic agents exist and the cancer cure rate has been considerably improved, the need for new synthetic drugs with possibly fewer side effects is still a fundamental issue.

Nowadays almost 60% of antineoplastic drugs used in Western medicine are metabolites of naturally occurring compounds [3]. Recently however, new technologies have made drug screening and synthesis of novel compounds with better performance properties easier and more effective.
Several of these newly investigated drugs appear to be very promising in their antineoplastic effects, as they are able to induce apoptosis and inhibit several different enzymes of DNA synthesis in a large number of cancer cell lines. Especially compounds with hydroxyguanidine, thiosemicarbazide and substituted benzohydroxamic acid functional groups are being reviewed [4].

In this diploma thesis a novel group of thiosemicarbazones (VG-series), which was synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, India, was investigated for their anticancer activity in the human HL-60 promyelocytic leukemia cell line.

A selection of the most promising compounds was reviewed for their cytotoxicity utilizing different growth inhibition assays. These compounds were also combined with

arabinofuranosylcytosine (Ara-C), a clinically well-established anticancer drug in leukemia therapy, and simultaneous as well as sequential growth inhibition assays were conducted. Cell cycle distribution effects were analyzed with a FACSCalibur flow cytometer.

Ribonucleotide reductase (RR) is the enzyme responsible for the reduction of ribonucleoside diphosphates into deoxyribonucleoside diphosphates. These are the chief components for DNA *de novo* synthesis and are thus crucial for the replication of DNA. RR is also responsible for the maintenance of the four deoxyribonucleoside triphosphate (dNTP) pools. Since RR activity is highly upregulated in tumor cells, it is believed to be a perfect target for cancer chemotherapy [5, 37].
A particular HPLC method was utilized in order to be able to determine if the most promising selected compounds affect the balance of dNTP pools.

Subsequent western blot analyses allowed for an improved understanding of the workings of growth inhibiting and apoptotic pathways [37].

This study sheds light on the biological activities of the above mentioned synthetically synthesized compounds and provides new information concerning their mode of action. Very promising results were obtained and further preclinical testing followed by *in vivo* studies is recommended.

2. Introduction

2.1. Cancer & Carcinogenesis

Cancer is a complex disease with over 100 distinguishable types and subtypes of tumors, which is marked by radical changes in the genome. Regulatory circuits, that are responsible for normal cell proliferation and homeostasis, are defect in cancer cells [6].
Carcinogenesis (the generation of cancer) is a multistep process where mainly two essential groups of genes are involved – oncogenes and tumor suppressor genes. Different mutations produce oncogenes that gain in function and tumor suppressor genes that lose part of their function. This process can transform normal human cells into malignant cancers [6, 7].

Tumor suppressor genes are indispensible, since they are able to block cell cycle progression when needed. (Proto)-oncogenes, in turn, activate cell proliferation and can cause uncontrollable growth [8].

The genomes of tumor cells are altered at multiple sites. These alterations can vary from one point mutation to several changes in chromosome complement.
Research suggests that six acquired modifications in cell physiology command malignant growth. These so-called hallmarks of cancer are: self-sufficiency in proliferative signals, indifference to growth-inhibitory signals, evasion of apoptosis, permanent replicative potential, continued angiogenesis as well as tissue invasion and metastasis.

Each of these mutations by itself or in combination, which develop during tumorigenesis, allow tumor cells to develop faster, uncontrollably and invade surrounding tissues. The body’s defense mechanism is most effectively breached [6].

Figure 1 exemplifies the capabilities acquired by cancer cells, which give them advantage over normal human cells.

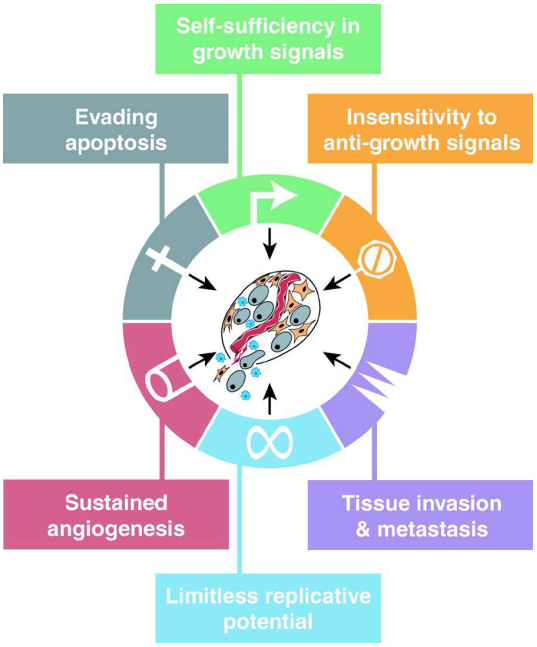

Figure 1: The hallmarks of cancer [6]

In addition to these six characteristics described by Hanahan and Weinberg, two new arising hallmarks are assumed to be involved in the pathogenesis of cancer. One pertains to the ability of cancer cells to alter or reprogram cellular metabolism and thereby endorse neoplastic growth. The other hallmark permits cancer cells to avoid immune destruction. This means that they are able to escape T- and B-lymphocytes, macrophages as well as natural killer cells. Since these characteristics have not been completely confirmed, they are pronounced emerging hallmarks [1].

2.2. Cell Cycle and Cancer

2.2.1. Cell Cycle

The only possibility our body has to produce a new cell is to duplicate an already existing cell. This means that all living creatures are made through continuing rounds of cell growth and division. Cells are able to duplicate their contents and then divide into two. This process is referred to as the cell cycle [7].

There are four phases in the eukaryotic cell cycle. During S-phase (DNA synthesis-phase) chromosomes are duplicated. Subsequently, chromosomes are segregated and the cells divided. This occurs in M-phase (mitosis). Since extra time for growth is needed the cell cycle has gap phases (G1 and G2) between mitosis and S-phase and between S-phase and mitosis respectively. These four phases (G1, S, G2 and M) combined are referred to as Interphase. Cell growth takes place throughout the cycle, exempting mitosis [7].

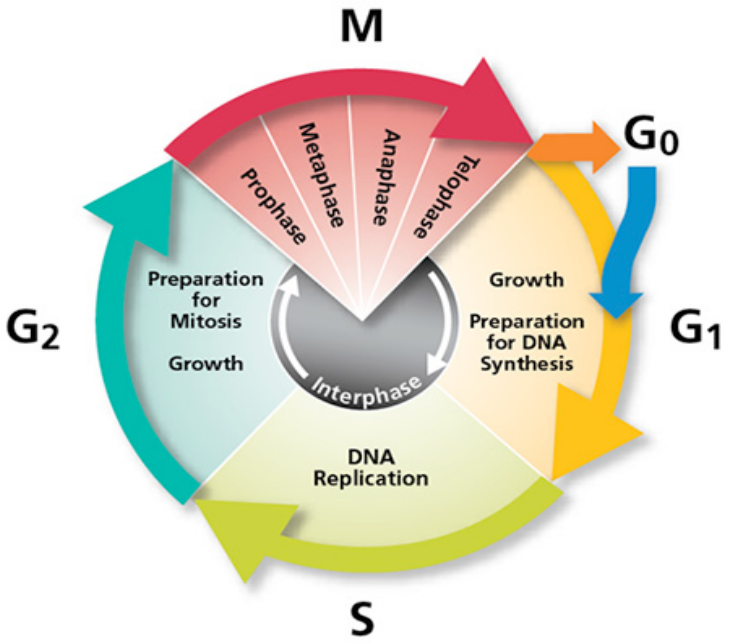

Figure 2: The cell cycle [9]

2.2.2. Cell Cycle Control

Different cellular proteins are responsible for the regulation of the transitions between the phases of the cell cycle. The cyclin dependent kinases (CDK), a family of regulatory proteins, are active at certain points during the cell cycle. When active, CDKs phosphorylate numerous proteins and initiate different downstream processes. Throughout the cell cycle, CDK protein expression remains stable, whereas cyclin protein levels rise and fall and in this way intermittently activate CDK.

Different cyclin proteins are needed in the different stages of the cell cycle [8]. In G1 phase cyclin D activates CDK4 or CDK6, in G1/S phase cyclin E forms a complex

with CDK2, in S phase cyclin A activates CDK2 and in M phase cyclin B is associated with CDK1 [7].
Several controls/checkpoints exist during the cell cycle to ensure that every phase is completed correctly before the next phase is entered. In G1 phase there is a restriction point, which is defined as a point of no return. This means that once this point is surpassed the cell must enter the cell cycle. If, at this point, the cell cycle is brought to a halt due to DNA damage, this stop is induced by the tumor suppressor protein p53. P53 has the ability to induce apoptosis by activating the transcription of certain genes (e.g. p21), if the damage is irreparable.

Checkpoints that are responsible for DNA damage detection are before S phase or after DNA replication. P21 can also arrest the cell cycle before damaged DNA is replicated.
Depending on the type of stimulation, these proteins can induce cell cycle arrest, cell death or cell proliferation [8, 10].

2.2.3. Loss of Cell Cycle Control

It becomes clear that all steps in the cell cycle are linked and that if only one key player is defective, the consequences for the cell can be disastrous. Defects in the proteins involved (CDKs, cyclins, checkpoint proteins) can lead to uncontrollable growth and in consequence to tumor development. However, understanding these connections between the cell cycle and apoptosis is not only helpful, but essential when researching new therapeutic possibilities [10].

2.3. Cell Death

It is possible to classify cell death according to different criteria. If classified by its morphological appearance, one can differentiate between apoptotic, necrotic or autophagic cell death. The Nomenclature Committee on Cell Death (NCCD) suggests that a cell is dead when the following processes have taken place: (a) the plasma membrane is no longer intact, (b) the cell is fragmented into discrete bodies and (c) its body has been engulfed by a neighboring cell [11].

2.3.1. Apoptotic cell death

Programmed cell death by means of apoptosis is vital in its purpose to protect against the development of cancer. In tumors, especially in those that progress to high grades of malignancy and are able to resist therapy, apoptotic effects are decreased.
When the apoptotic regulatory system is activated a cascade of events takes place. Caspases induce proteolysis; the cell is gradually disassembled and subsequently consumed by its neighboring cells as well as by phagocytes.
The proteins involved in this regulation act either as inhibitors (e.g. B-cell lymphoma 2; Bcl-2) or as initiators (e.g. Bax and Bak) of apoptosis. Bcl-2 binds to Bax and Bak (proapoptotic triggering factors), thus suppressing apoptosis. When Bax and Bak are not bound to Bcl-2, they are able to disrupt the outer mitochondrial membrane integrity, which in turn causes the release of proapoptotic signaling proteins (e.g. cytochrome c). A cascade of caspases is set off and apoptosis can be initiated [1].
Tumor cells develop different strategies that allow them to evade apoptosis. Most commonly the tumor suppressor protein p53 loses its function, thus the ability to start programmed cell death is lost. Furthermore, the expression of antiapoptotic regulators is increased in tumor cells, whereas the expression of proapoptotic factors is downregulated [1].

2.3.2. Necrotic cell death

Necrotic cell death, in comparison with apoptotic cell death, is not linked to the activation of caspases and is usually followed by inflammatory reactions. In necrotic cells the organelles swell, the plasma membrane ruptures and ultimately the cell lyses. Its contents are released into the local tissue [12].
The proinflammatory signals that are released after this cell death allow necrotic cells to recruit inflammatory cells. Their function is to examine the damage and remove the necrotic remains. Evidence suggests however, that these inflammatory cells can promote tumors, since they are able to further angiogenesis and cell proliferation. Furthermore, necrotic cells can release factors such as IL-1α (Interleukin 1-alpha), which can stimulate neighboring cells to proliferate and promote neoplastic growth [1].

2.3.3. Autophagic cell death

Autophagic cell death, a vital cell-physiological response, is considered a self-cannibalization method. It encompasses the envelopment of cytoplasmic material and break down of cellular organelles with the help of intracellular vesicles called autophagsomes. The autophagosomes then fuse with lysosomes (autolysosomes), wherein the material is degraded.

In order to maintain healthy cells a certain level of autophagy is needed. Environmental factors, for example, such as starvation or nutrient deprivation can activate autophagy. This means that autophagic cell death is induced in response to cellular stress and is essential for survival. Yet even though the autophagic program is cytoprotective, it can promote cell death in healthy cells as well as in tumor cells. Exuberant autophagy can be very damaging [1, 12].

2.4. Leukemia

2.4.1. Introduction and Classification

Leukemia is a malignant type of cancer that originates in blood and in blood forming tissue. It is a hematologic neoplasm, where the bone marrow produces abnormal white blood cells that enter the blood stream. Unlike normal blood cells, leukemia cells do not die and so they accumulate and crowd out healthy cells [13].
In a healthy person hematopoietic stem cells produce all types of blood cells. Stem cells have the unique ability to repair themselves and to differentiate into various cell types. These traits enable hematopoietic stem cells to restock the whole blood system. Therefore even a single mutation can cause drastic problems. Mutations that induce excessive growth or that arrest growth of partially differentiated cells are the leading cause of leukemia [14, 37].

Myeloid stem cells are responsible for the production of red blood cells and platelets, whereas lymphoid stem cells produce white blood cells. Depending on the kind of

blood cells affected (myeloid or lymphoid), as well as the progression of the disease, one can differentiate between various types of leukemia [13].
Malignant leukocytes that are of myeloid origin are granulocytes, monocytes and erythrocytes. Malignant leukocytes of lymphoid origin are B- and T-cells as well as natural killer cells [15].

When distinguishing according to the progression of the disease, one can divide in acute and chronic leukemia. Chronic leukemias (chronic myeloid leukemia = CML, chronic lymphocytic leukemia =CLL) are often only found during a routine check up, since patients can live for years without any symptoms. The patients show highly increased levels of abnormal white blood cells (=disproportionate proliferation of mature blood cells).
Acute leukemias (acute myeloid leukemia = AML, acute lymphocytic leukemia = ALL) develop very rapidly and therefore symptoms are noticed far earlier than in chronic leukemias. Symptoms include infections, fatigue, anemia, petechiae and easy bleeding. The disease is characterized by a large increase in immature abnormal myeloid or lymphoid cells in the bone marrow [16, 13].

To this day the exact causes for developing leukemia are not completely clear. There are however, several risk factors, which can increase the chances of falling ill. These factors include exposure to radiation, previous chemotherapy or radiation therapy or having certain congenital genetic syndromes.

Treatment is dependent on the type of leukemia, the progression of the disease as well as on the age of the patient. Complete remission (CR = reduction of the total body leukemic cell population from ~10^{12} to 10^{9} cells) is strived for [17].
Therapy options also include chemotherapy, radiation therapy and possibly stem cell transplantation (SCT) [13].

2.4.2. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) can also be referred to as acute myelocytic leukemia, acute myelogenous leukemia, acute granulocytic leukemia or acute non-lymphocytic leukemia. The disease affects all age groups. However, the median age of patients

diagnosed with AML is about 65 and although it is the most common type of leukemia in adults, the survival rates of older patients are very low. Comorbidities connected to ageing are thought to be responsible for this fact [18, 19].

Acute myeloid leukemia (AML) is a clonal disorder, in which immature progenitor cells (so-called blasts) are rapidly overproduced. The cells lose the ability to differentiate normally and normal hematopoiesis is no longer possible. This deficit typically leads to bleeding, infection, organ infiltration (usually lungs and brain) and ultimately to death within a year if left untreated. The cause of death is mostly bone marrow failure.
Several risk factors are associated with the outbreak of AML. They include exposure to radiation, benzene and cytotoxic chemotherapy. The majority of cytogenetic abnormalities that lead to AML are translocations between chromosomes 15 and 17, t(15;17), (a characteristic translocation found in acute promyelocytic leukemia), between chromosomes 8 and 21, t(8;21) and anomalies in the long arm of chromosome 11 (11q) [19].

2.4.2.1. Treatment Options

In the last three decades AML has been treated with chemotherapy using different combinations of anthracyclines such as daunorubicin or idarubicin and arabinofuranosylcytosine (Ara-C). Ara-C (a deoxycytidine analogue/ antimetabolite) is integrated into nascent DNA. In order for cells to progress from G1 to S phase a series of activations of different cyclins by phosphorylation is needed. Ara-C inhibits DNA replication in leukemic cells at this G1/S phase checkpoint and therefore hinders the cells to complete the cell cycle [19, 20].

AML treatment commonly takes place in two phases. In the first phase one strives for complete remission, which means bone marrow with less than 5% blasts, a neutrophil count greater than 1000 and a platelet count greater than 100 000. In the second phase of treatment the aim is to extend the complete remission for as long as possible. After three years of remission the probability of a relapse is only around 10% [19].

Depending on a patient's cytogenetic material the risk of AML can be characterized as favorable, intermediate or adverse. Accordingly the cure rates are completely diverging. Furthermore, every year after the age of 18 that a patient falls ill with AML worsens the prognoses. Studies show that chances of poor outcomes, resistance to therapy and death are more often seen in elderly patients. It appears that unknown genetic and biological factors might be responsible [19].

2.4.2.2. New Possibilities

Regrettably Ara-C is not equally effective in all patients. This can be due to drug resistance/CdR-kinase deficiency (deoxycytidin kinase), inactivation of Ara-C by Cr-deaminase (cytidine deaminase), high frequency of leukemic stem cells and respectively patients with adverse cytogenetics [20].

However, in recent years a lot of energy has been invested in research on finding new antineoplastic agents for AML. New drugs (e.g. small molecule inhibitors) that target signaling pathways as well as cell surface molecules on leukemic cells are being examined. Furthermore, substances that show amplifying effects on already existing antineoplastic agents are also being tested.
The most common mutation found in AML is a mutation concerning the FMS-like tyrosine kinase 3 receptor (Flt3 receptor kinase). The receptor is expressed on hematopoietic stem cells and regulates stem cell differentiation and proliferation. In vitro tests have shown that receptor activation both causes proliferation of AML cells and inhibits apoptosis of AML cells. The occurrence of Flt3 receptor mutations increase with age and studies suggest that these mutations are responsible for bad prognoses. This fact has resulted in a search for new Flt3 tyrosine kinase inhibitors as well as other enzyme inhibitors in this signaling pathway [21].

2.4.3. Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is considered the most curable subtype of acute myeloid leukemia, although more than 20% of patients with APL die from the disease [21]. It is an illness most commonly diagnosed in young adults or in patients over 60.

APL is caused by a translocation between chromosomes 15 and 17 [t (15;17)]. This translocation can take place, because both chromosomes break. Chromosome 15 dislocates the promyelocytic leukemia (PML) gene, which is responsible for encrypting a growth suppressing transcription factor. Chromosome 17 disrupts the retinoic acid receptor alpha (RARα) gene, which is in charge of myeloid differentiation. A PML/RARα fusion gene is generated that is able to produce a chimeric protein, which in turn stops the maturation of myeloid cells at the promyelocytic stage. An increased proliferation of promyelocytes is the consequence [22].

Treatment options for patients with APL include a combination therapy of all-trans retinoic acid (ATRA) or arsenic trioxide (ATO) with anthracycline chemotherapy. These agents target the oncogenic transcription factor promyelocytic leukemia (PML)-retinoic acid receptor alpha (RARα) and are believed to be involved in the elimination of leukemia stem cells [15].

2.4.4. *In vitro* model

The human HL-60 cell line is an acute promyelocytic leukemia (APL) cell line. The peripheral blood leucocytes that were used for all the experiments in this thesis were obtained from a 36-year-old Caucasian woman with acute promyelocytic leukemia. (www.atcc.org)
This *in vitro* model has been widely used especially when examining ribonucleotide reductase activity after incubation with various drugs [4].

2.5. Ribonucleotide Reductase

2.5.1. General Information

Ribonucleotide reductase (RR) is the rate-limiting enzyme for the *de novo* synthesis of deoxyribonucleotides (dNTPs). Peter Reichard first reported its discovery in E. Coli in 1961 [23]. RR catalyzes the reduction of all four ribonucleoside diphosphates into 2'deoxyribonucleoside diphosphates, therefore supplying the cell with forerunners for DNA synthesis and repair [24, 25]. And even though the structure, catalytic and

many inhibitory mechanisms were already uncovered, many questions have yet to be answered [23].

Genetic information in the form of DNA is part of all cellular life forms. Genomic screenings have confirmed that RR can be found in all growing cells of all living organisms. Even various virus species have functioning RRs in order to ensure quicker proliferation in infected host cells [25].
Since malignant tissues have an amplified need of deoxyribonucleoside triphosphates, RR is especially active here in comparison to normal tissues/cells. RR inhibition has been a key target for therapeutic intervention in numerous diseases with abnormal cell proliferation. It is considered an excellent target for cancer chemotherapy [4, 23].
In order to function properly it is vital for all living cells that the pool of deoxyribonucleoside triphosphates (dNTPs) is balanced. The allosteric control of the substrate specificity of RR is crucial for pool regulation. Unequal production of dNTPs increases the likelihood of mutations, which in turn can cause malignant cell growth [26].

2.5.2. Classes of RRs

Ribonucleotide reductases can be divided into three main classes depending on their metallo-cofactor for radical generation. Class 1 enzymes are found in prokaryotes, eukaryotes and viruses. They are 1:1 protein complexes, made up of two homodimers, R1 and R2. The larger homodimer, R1, has numerous binding sites for regulation and catalysis. R2, the smaller homodimer, contains a tyrosyl free radical and a diiron center vital for nucleotide reduction. Class 2 enzymes are found mainly in bacteria. They have a transient 5'-deoxyadenosyl radical. Class 3 enzymes are found mainly in anaerobic bacteria and contain a stable glycyl radical [27].
All three classes of enzymes have a conserved cysteine residue at the active site in common that is converted into a thiyl radical. This radical is responsible for abstracting a hydrogen atom from the ribose ring of the substrate [25].

Human ribonucleotide reductase is a class 1 enzyme, which depends on the presence of oxygen to function properly [25]. It is a tetramer ($\alpha_2\beta_2$) and has two homolo-

gous subunits, hRRM2 and p53R2. The p53R2 subunit is regulated by the tumor suppressor protein p53. P53 is fundamental in the G1-G2 phase of the cell cycle in that it regulates nucleotide supply during DNA repair and controls apoptosis. Research suggests that p53R2 and p53 form a protein-protein complex. In cellular stress situations p53 no longer binds to p53R2 and induces transcription of p53R2 for later use. Binding p53R2 to R1 initiates DNA repair [27].

Even though the sequence homology and oxygen needs of the three classes of RRs are very diverse, they share basic similarities in the overall structure of their active sites. They also share an identical allosteric regulation of substrate specificity. Based on these structural semblances, it is supposed that all classes of RRs evolved divergently from a common ancestor. The different mechanisms that take place in radical creation are assumed to have developed later due to environmental selective pressure [26].

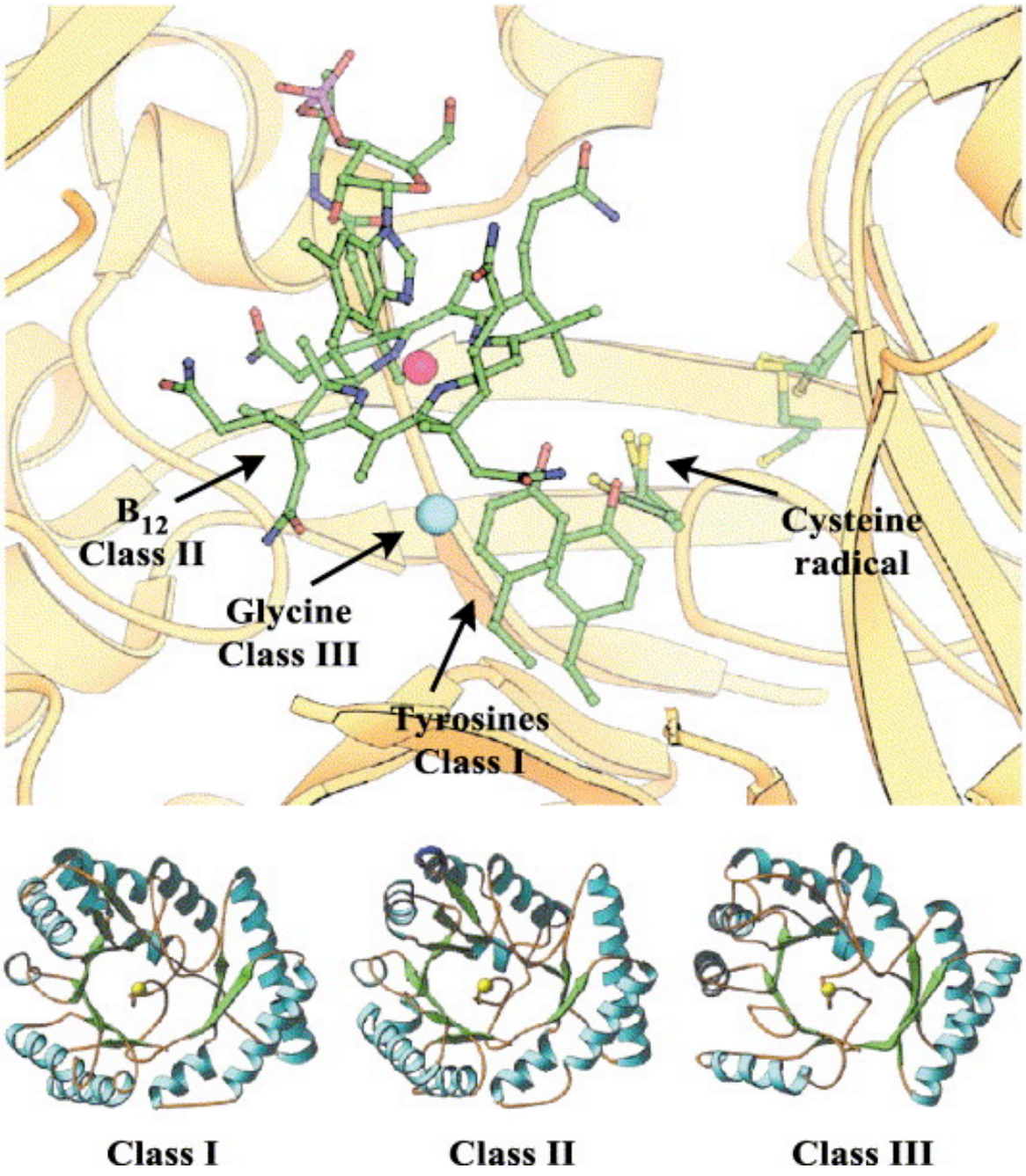

Figure 3: Substrate active binding sites in the three RR classes [25]

2.5.3. RR Structure

The exact structure of ribonucleotide reductase has still not been completely clarified. Particularly the tyrosyl diiron reaction center in the R2 subunit still leaves several questions unanswered. The entire evidence concerning the structure of the R2 subunit as well as the purpose of the redox center is based on the findings from studying the Escherichia coli ribonucleotide reductase. It is possible to compare the RRs,

since murine, human and E. coli RRs are all class 1 enzymes. With the help of the E. coli RR model it could be proven that the dinuclear iron cluster in R2 produces a stable tyrosyl radical. After being transferred to the R1 catalytic site, the radical plays a central role in deoxynucleotide formation [27].

The R1 subunit contains both activity and allosteric sites. The substrate site is situated in the center of each monomer with the allosteric specificity site nearby. Yet the distance between the substrate and the effector sites is too far to allow (direct) communication between substrate and effector. Consequently the effector signal to initiate a reaction at the catalytic site must happen via the protein structure. The allosteric activity site is found in a crevice made by 100 N-terminal amino acids of each monomer [26].

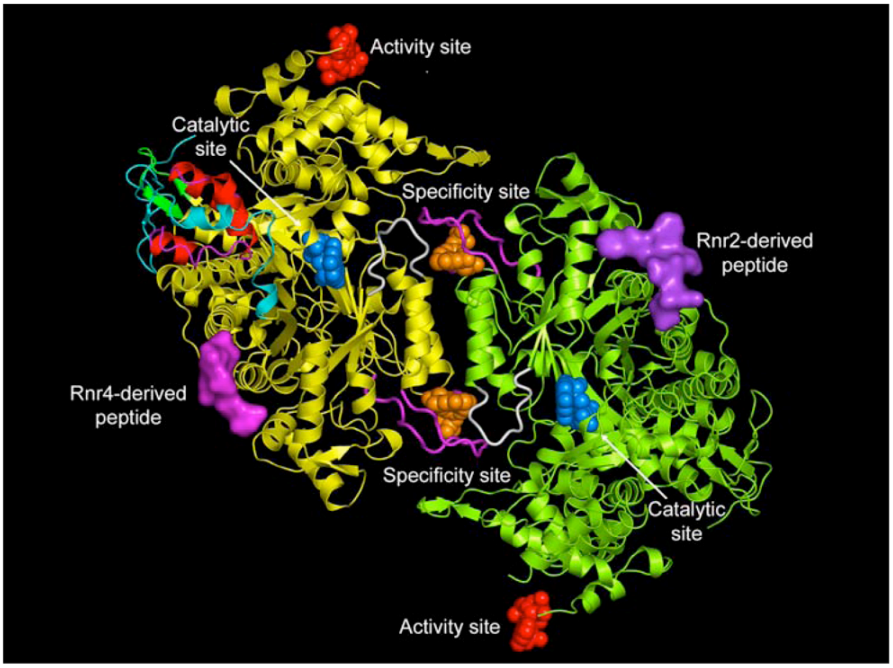

Figure 4: Class 1 Ribonucleotide reductase [28]

2.5.4. Allosteric Regulation

Ribonucleotide reductase has two separate allosteric sites. Each one has a particular function. The A-site (activity site) is responsible for the catalytic activity and the S-site (specificity site) determines the nature of the substrate to be reduced in order to produce deoxyribonucleotides. This allows the enzyme to control the amounts of dNTPs produced for DNA replication and furthermore adapt to changes (more or less de-

mand for dNTPs). Both ATP and deoxynucleoside triphosphates are able to function as allosteric effectors.

The A-site works as a secondary allosteric regulatory site. It is in charge of regulating the on- and off-switch of the enzyme. Depending on the effector, the enzyme is either switched on or off. If ATP binds, it activates RR, whereas when dATP binds, RR is inhibited. This means that while dATP exerts inhibiting effects on the A-site, it acts stimulating on the S-site. Conversely, the affinity of dATP to the S-site is greater than to the A-site and this feedback regulation allows the cell to control the dNTP pool levels [26].

2.5.5. Catalytic cycle in Class 1 RRs

In the early seventies it was documented that the cysteine residues are involved in the reduction of ribonucleotides. It was also recognized that the tyrosyl radical in R2 was not directly responsible for catalysis, but a different radical at the active site of R1 had to be liable for abstracting the hydrogen from the substrate. Analysis showed that this radical had to be on the conserved cysteine residue C439.

To this day, the complex mechanism of the substrate turnover in class 1 RRs is not completely understood. The facts that are known include that a complex is formed between R1 and R2 and that the substrate is bound to R1 in its reduced form since the tyrosyl radical in R2 needs to be transferred to the active site of R1.
In a first step the thiyl radical C439 abstracts the 3'-hydrogen atom from the ribose ring of the substrate. This creates a substrate radical. This in turn makes the 2'OH-group more available to acid catalysis. It is protonated by C225 and subsequently leaves as a water molecule. The substrate is now a 2'-ketyl radical. In a next step the two cysteine residues (C225 and C462) are oxidized while transporting a hydrogen atom to the substrate. Subsequently, the excess radical electron is transported to the substrate 2'-position via a chain of hydrogen bonded active site residues. This re-generates a substrate radical. The substrate radical abstracts the hydrogen from C349 and this in turn finalizes the production of the deoxyribonucleotide and a thiyl radical on C349. The radical state is transported back to the R2 subunit via a radical transfer chain and this in turn restores the stable tyrosyl radical [25].

2.5.6. RR in Cancer

Ribonucleotide reductase is notably upreglated in rapidly proliferating tumor cells, as they have a much greater need of dNTPs for *de novo* DNA synthesis. Recent studies have shown that differences in enzyme activity between fast and slow growing tumor cells are almost 200-fold [29]. Consequently, inhibitors that specifically target RR are used in cancer chemotherapy. Since rapidly proliferating cancer cells are predominantly targeted, these inhibitors are especially advantageous in treatment of malignant diseases [4, 30].
Widely known and used RR inhibitors include hydroxyurea (HU), fludarabine or difluorodeoxycytidine (Gemcitabine, dFdC). They have been especially successfully applied in combination therapy against leukemia or pancreatic cancer. Hydroxyurea, the first clinically used RR inhibitor, has shown great synergistic effects in combination with Ara-C [4, 25].

RR inhibitors are capable to function as radical scavengers or as metal chelators. The R2 subunit of RR has two dinuclear iron centers, each stabilizing a tyrosyl radical (see above). If radical scavenging or iron chelation inhibits the R2 subunit, the enzyme as a whole is destabilized. Subsequently, RR inhibitors cause disparities in dNTP pool levels [4].

2.6. Thiosemicarbazones

2.6.1. General Information

Owing to their antiviral, antibacterial and antitumor activity, thiosemicarbazones and their derivatives have called attention to themselves in the fields of chemistry, biology and pharmacology. They are metal ion chelators and several of their biological functions are accredited to their capability to form biologically active complexes. Both thiosemicarbazones and as their metal complexes have been reviewed particularly for their antineoplastic effects. Earlier studies showed that copper complexes with aromatic thiosemicarbazones have a distinct effect on the modalities that cause leuke-

mic transformation. They are able to inhibit replication and trigger apoptosis, therefore operating as potential antineoplastic agents [31, 32].

2.6.2. Pharmacological Properties and Synthesis

The pharmacological properties of thiosemicarbazones and bis(thiosemicarbazones) are directly associated to their capability to chelate transition metals such as Zn(II), Fe(II)/(III) and Cu(II) and thus form lipophilic or neutral complexes. Even small variations in structure can cause major changes in metal complex stability, redox potentials, membrane permeability and even biological activity.
In order to synthesize a thiosemicarbazone, a condensation reaction has to take place between a thiosemicarbazide and an aldehyde or ketone. Thiosemicarbazones can also be synthesized by letting a methyl ester of imidopicolinic acid and a thiosemicarbazide react.

Thiosemicarbazone structures contain a sulfur atom and an azomethinic nitrogen atom (also known as *Schiff base*), which both can function as chelating ligands. They form a five membered ring, which can chelate transition metal ions. They also contain hydrazinic nitrogen, which can deprotonate after complexation. This causes a bond delocalization and lengthening of the C-S bond. Therefore, the thione groups appear to be “masked” thiolate groups [33].

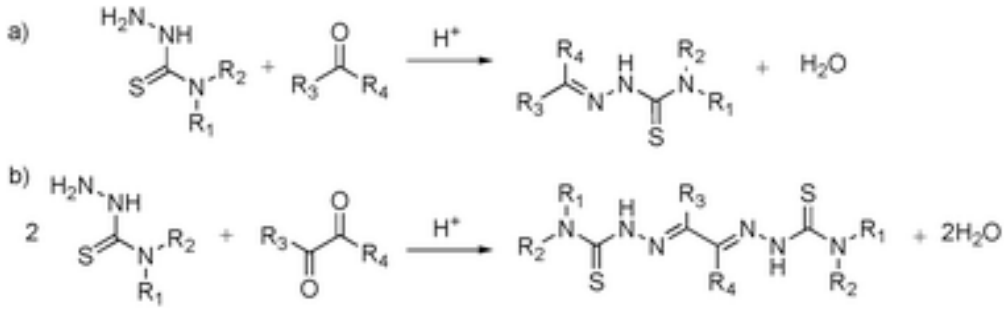

Figure 5: Synthesis of (a) thiosemicarbazones and (b) 1,2-bis(thiosemicarbazone) via condensation [33]

2.6.3. Thiosemicarbazones and Cancer

Tumor cells are characterized by an abnormally high proliferation rate as well as their sensitivity to iron deprivation. Being an essential element that is compulsory for many cellular processes (e.g. cell proliferation), iron deficiency can have dramatic effects on proliferating tumor cells. Thus, in the last years, iron chelation therapy has been looked at more closely as a possible treatment option against various tumors. The elevated need for iron in cancer cells is noticeable due to the amplified expression of the transferrin receptor 1 (TfR1). This receptor is responsible for the iron uptake from the iron transport protein transferrin (Tf) on the cellular surface. This and the fact that RR is expressed distinctly higher, make tumor cells very sensitive to iron chelation.

The drugs with the most effect and promise in battling advanced leukemia are thiosemicarbazone-based agents. Ribonucleotide reductase (RR) is the focal target of these thiosemicarbazones. If RR is inhibited, dNTP pools are diminished and DNA synthesis is disrupted.
Triapine (3-aminopyridine-2-carboxyldehyde thiosemicarbazone), a very strong RR inhibitor, was the first thiosemicarbazone used in clinical practice and was even clinically evaluated. Even though it showed very promising effects against advanced leukemia, it was ineffective against various solid tumors.

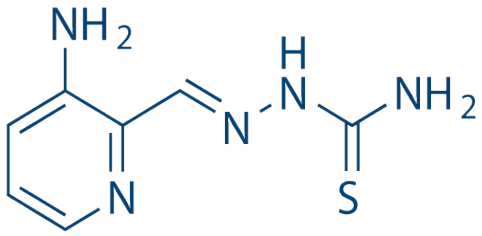

Figure 6: Triapine [34]

Evidently, there is a great need to learn more about the molecular mechanisms of thiosemicarbazones in anticancer activity. The resistance development of cancer cells against these novel agents is also a primary focus of upcoming research pro-

jects. Accordingly new RR inhibitors and new agents with higher tumor selectivity and activity are being developed and studied [35].

2.6.4. Mode of Action

Various novel thiosemicarbazone chelators have been examined as prospective anti-tumor agents. However, the exact mode of operation of these agents has not yet been completely clarified. The mechanisms that are known include: the inhibition of RR activity and of cellular iron uptake via transferrin; the mobilization of iron from cells; the formation of redox-active metal complexes that produce reactive oxygen species (ROS) and the upregulation of metastasis suppressor protein [36].

2.6.5. VG-Series

In order to find connections between chemical structures or structure related properties and biological activities of novel compounds, modern science uses qualitative and quantitative structure-activity relationship (QSAR) studies. On the basis of the best QSAR model, 40 novel compounds with potential ribonucleotide reductase inhibiting properties were synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, India. Preliminary testing (growth inhibition assays) was conducted on all substances. Cell counts and IC_{50} values were determined after 24, 48 and 72 hours and the 12 substances with the lowest IC_{50} values were chosen for further testing.
In turn, three of these agents were elected to be analyzed based on their activity in human HL-60 promyelocytic leukemia cells and VG-22 was chosen to be analyzed in more depth as it seemed to have the most promising properties out of the three.

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals and supplies

The thiosemicarbazones (VG-series) were synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, India. Arabinofuranosylcytosine (Ara-C) and solvent DMSO were obtained from Sigma-Aldrich GmbH, Vienna, Austria and were of highest purity available.
All other chemicals and reagents used were commercially available and of highest purity.
The following reagents, chemicals and materials were purchased as described below [37]:

Acetonitrile	J.T. Baker
Ammoniumdihydrogenphosphate	VWR Merck, Vienna, Austria
Cellpack buffer	SYSMEX, Europe GmbH
Chloroform: isoamyl alcohol 24:1	SIGMA, St. Louis, MO
Cytidine-2-14-C (56 Ci/mmol)	SIGMA, St. Louis, MO
Deoxyadenosine triphosphate	SIGMA, St. Louis, MO
Deoxycytosine triphosphate	SIGMA, St. Louis, MO
Deoxyguanosine triphosphate	SIGMA, St. Louis, MO
Deoxythymidine triphosphate	SIGMA, St. Louis, MO
Ethanol	SIGMA, St. Louis, MO
Freon	SIGMA, St. Louis, MO
Guanisine triphosphate	SIGMA, St. Louis, MO
Phenol: chloroform: isoamyl alcohol 25:24:1	SIGMA, St. Louis, MO
L-Rhamnose	SIGMA, St. Louis, MO
Methylamine	SIGMA, St. Louis, MO
Phenol	SIGMA, St. Louis, MO
Phosphoric acid	VWR Merck, Vienna, Austria
PIC (Protease inhibitor cocktail)	SIGMA, St. Louis, MO

Pierce ECL Western Blotting substrate	Thermo Scientific, Rockford, IL
PMSF (Phenyl Methyl sulfonic fluoride)	SIGMA, St. Louis, MO
Ponceau S solution	SIGMA, St. Louis, MO
Propidium iodide	SIGMA, St. Louis, MO
Proteinase K	Roche, Vienna, Austria
RNAse A	Amersham Pharm., Vienna, Austria
Sarcosine	SIGMA, St. Louis, MO
Sodium acetate	Life Technologies, Paisley, Scotland
Sodium periodate	SIGMA, St. Louis, MO
Trichloroacetic acid	SIGMA, St. Louis, MO
Tri-n-ocylamine	SIGMA, St. Louis, MO
Trizma base	SIGMA, St. Louis, MO
TRIS/EDTA buffer	SIGMA, St. Louis, MO
Triton-X-100	SIGMA, St. Louis, MO
Tween 20	SIGMA, St. Louis, MO

3.1.2. Cell culture media and supplements

RPMI 1640 Medium with L-Glutamine	PAN Biotech
Fetal Calf Serum (FCS), heat inactivated	PAN Biotech
Penicillin/Streptomycin 10.000 U/ml solution	PAN Biotech
L-Glutamine 200mM solution	GE Healthcare

3.1.3. Cell line

The human HL-60 promyelocytic leukemia cell line was acquired from the American Type Culture Collection, Manassas, VA, USA (ATCC). The cell line was maintained in RPMI-1640 medium, which was supplemented with 10% heat inactivated fetal calf serum (FCS), 1% Penicillin/Streptomycin and 1% L-Glutamine at 37°C in a humidified atmosphere containing 5% CO_2 using a Heraeus Cytoperm 2 incubator (Heraeus, Vienna, Austria).
Cells were grown in 25cm^2 tissue culture flasks and counted using a microcellcounter CC-110 (SYSMEX, Kobe, Japan).

Only cells in the logarithmic growth phase were used for all the subsequently described experiments [4].

3.1.4. Devices

BD 6220 Incubator	Heraeus, Vienna, Austria
Biometra WT12	CORE Life Sciences
Bioshake IQ	Q.Instruments GmbH
Centrifuge 5415R	Eppendorf
Hettich Rotanta Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany
Hitachi U-200 Double Beam Spectrophotometer	Hitachi, Austria
Laminar Air Flow	Dipl.Ing. W. Ehret GmbH., Emmendingen, Germany
Microcellcounter CC-110	SYSMEX, Kobe, Japan
MiniSpinPlus	Eppendorf
Magnetic Stirrer	Framo, Eisenbach, Germany
Mettler PJ 300/AT 250 scales	Mettler, Vienna, Austria
Olympus IMT-2 inverse microscope	Olympus, Vienna, Austria
PH-Meter	Metrohm, Switzerland
Vakuumierer VC10	Caso, Germany
Wallac 1414 Liquid Scintillation Counter	PerkinElmer, Boston, MA
Water bath	Gesellschaft für Labortechnik mbH, Burgwedel, Germany

3.2. Methods

3.2.1. Growth inhibition assay

Logarithmically growing HL-60 cells were cultivated in 25cm^2 tissue culture flasks ($0.1x10^6$ per ml) and incubated with increasing concentrations of drugs at 37°C under cell culture conditions. Cell counts and IC_{50} values (half maximal inhibitory concentra-

tion/concentration of drugs needed for 50% inhibition) were determined after 24, 48 and 72 hours using the microcellcounter CC-110. Results were calculated as the number of viable cells. All experiments were performed in triplicates [4].

3.2.1.1. Simultaneous growth inhibition assay

HL-60 cells ($0.1x10^6$ per ml) were simultaneously incubated with various concentrations of different Thiosemicarbazones and Ara-C at 37°C under cell culture conditions. Cell growth was documented after 24, 48 and 72 hours using the microcellcounter CC-110 [4].

3.2.1.1. Sequential growth inhibition assay

HL-60 cells ($0.1x10^6$ per ml) were treated with a single concentration of one of the thiosemicarbazones and incubated for 24 hours at 37°C under cell culture conditions. The cells were then counted, the compound was washed out and suspended in new RPMI-medium. In a next step, the cells were exposed to different concentrations of Ara-C and incubated for another 72 hours. Cell growth was documented after 24, 48 and 72 hours using the microcellcounter CC-110 [4].

3.2.2. Cell cycle distribution analysis

HL-60 cells ($0.4x10^6$ per ml) were cultivated in $25cm^2$ tissue culture flasks and incubated with increasing concentrations of drugs at 37°C under cell culture conditions. After 24 and 48 hours cells were harvested and suspended in 5ml cold PBS. Following this step the cells were centrifuged at 1000rpm for 5 minutes, once again suspended in PBS and then fixed in 1ml cold ethanol (70%) for 30 minutes at 4°C. After another two washing steps in cold PBS, 50µg/ml RNAse A and 50µg/ml propidium iodide was added to the cells before they were incubated at 4°C for 60 minutes. The cells were subsequently analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT Software (Verity Software House, Topsham, ME, USA) [4, 5].

3.2.3. Determination of deoxyribonucleoside triphosphates (dNTPs)

Garrett and Santi established this method used for extracting cellular dNTPs. HL-60 cells were cultivated in 175cm^2 tissue culture flasks and incubated with increasing concentrations of drugs for 24 hours. The cells were then counted and only 1x10^8 cells were used for dNTP extraction. Cells were centrifuged at 1500rpm at 4˚C for 5 minutes and suspended in 1ml ice-cold phosphate buffered saline (PBS). In a next step, the cells were lysed through addition of 10µl of trichloroacetic acid (TCA) and vortexed for 1 minute. After resting on ice for 30 minutes, the proteins were separated out of the lysate by centrifugation at 15000rpm at 4˚C for 10 minutes in an Eppendorf microcentrifuge. The supernatant liquid containing the dNTPs was removed and neutralized by adding 200µl Freon/Tri-N-octylamine (TNO) (7.8T Freon + 2.2T TNO). 30µl of methylamine solution (4M=270g/l; pH 7.5) and 10µl of sodium periodate solution (c=100g/l) was added for periodating the 100µl aliquots. The aliquots were then incubated at 37˚C for 30 minutes and when 5µl of 1M rhamnose solution was added, the reaction was terminated. The extracted dNTPs were measured using a Merck “La Chrom” HPLC system equipped with an L-7200 autosampler, L-7100 pump, L-7400 UV detector and D-7000 interface. Samples were eluted with 3.2M ammonium phosphate buffer, pH 3.6 (pH is adjusted by adding 0.32mol/l H_3PO_4), containing 20mM acetonitrile using a 4.6x250mm Parsitil 10 SAX analytical column (Whatman Ltd., Kent, UK). The separation was performed at room temperature with a flow rate of 2ml/min. The dNTP concentration was calculated as percent of total area under the curve for each sample [4, 5].

3.2.4. Western Blotting

HL-60 cells were cultivated in 25cm^2 tissue culture flasks at a concentration of not more than 0.5x10^6 per ml medium and incubated with a specific concentration of drugs (IC_{50} value) for 0.5, 2, 4, 8, and 24 hours.
After this first incubation period cells were harvested, counted and washed twice with ice-cold PBS (pH 7.2) and subsequently lysed using a mixture of 150mM NaCl, Tris-buffered saline (Tris pH 8.0), 1% Triton X-100, 1mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (PIC; 100x Stock). The lysates were centri-

fuged at 12000rpm for 20 minutes at 4˚C and the supernatants were temporarily stored at -20˚C.

Equal amounts of protein samples (20µl lysate per slot) were loaded into the wells of the SDS-PAGE gel. The proteins were electrophoresed at 120V (50mA) for roughly one hour and then electroblotted at 95V at 4˚C for 80 minutes onto PVDF membranes (Hybond P, Amersham, Buckinghamshire, UK).

To ensure that samples were loaded equally, the membranes are first stained with Ponceau S. After electroblotting the membranes were washed with TBS, and blocked in blocking solution (5% non-fat dry milk in TBS containing 0.1% Tween-20) for 60 minutes.

The membranes were then incubated with the first antibody (in a blocking solution, diluted 1:1000 or 1:5000), shrink-wrapped in a plastic film and stored overnight at 4˚C. After this step the membranes were washed with TBS/Tween-20 and incubated at room temperature for 60 minutes with the secondary antibody (peroxidase-conjugated goat anti-rabbit IgG or donkey anti goat IgG; diluted 1:1000 or 1:5000 in TBS/T). Chemoluminescence was developed using the ECL detection kit (Amersham, Buckinghamshire, UK) and subsequently, membranes were exposed to Amersham Hyperfilms [4, 5].

3.2.4.1. Antibodies

Chk1	#2345, polyclonal (Cell Signaling)
R1 (T-16)	#sc-11733, polyclonal (Santa Cruz)
p53R2 (N-16)	#sc-10840, polyclonal (Santa Cruz)
R2 (I-15)	#sc-10848, polyclonal (Santa Cruz)
Donkey anti-goat IgG-HRP	#sc-2020, (Santa Cruz)
Anti-rabbit IgG-HRP	#P0217, polyclonal (Dako)

3.2.5. Statistical Calculations

Dose-response curves were calculated using the Prism® 5.01 software package (GraphPad, San Diego, CA, USA) and statistical significance was determined by unpaired t-test ($P<0.05$ is considered significant). The calculations for the dose response curves and combination effects were performed by the Calcusyn® software

designed by Chou and Talalay (Biosoft, Ferguson, MO). This analytical method is used to describe the interaction amongst the different drugs in certain combinations. A combination index (CI) of 0.9-1.1 indicates additive effects of two inhibitors, a CI of <0.9 demonstrates synergism and a CI of >1.1 shows antagonism [4, 5].

4. Results

4.1. VG-10, VG-13, VG-22

4.1.1. Preliminary screening

HL-60 cells were cultivated in 25cm^2 tissue culture flasks (0.1x10^6 per ml) and incubated with increasing concentrations of novel thiosemicarbazones at 37°C under cell culture conditions. Twelve of the 40 substances of this series were deemed promising upon determining cell counts and IC_{50} values after 24, 48, and 72 hours.
Further growth inhibition experiments revealed VG-10, VG-13, and VG-22 to be the most potent compounds in this regard, which is why they were chosen for further investigation (figures 7a-c).

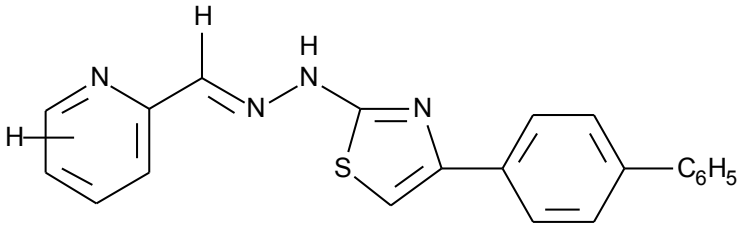

Figure 7a: Structural formula of VG-10

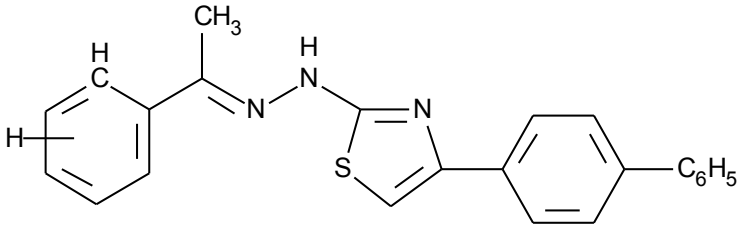

Figure 7b: Structural formula of VG-13

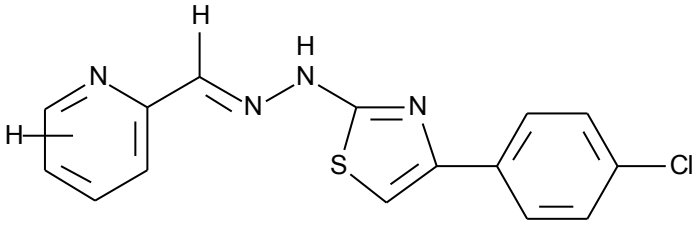

Figure 7c: Structural formula of VG-22

4.1.2. Influence of VG-10, VG-13, and VG-22 on the growth of HL-60 cells

Logarithmically growing HL-60 cells were cultivated in 25cm^2 tissue culture flasks ($0.1x10^6$ per ml) and incubated with increasing concentrations of VG-10 (2, 4, 6, 8, and 10µM) VG-13 (2, 4, 6, 8, and 10µM) or VG-22 (500nM, 1, 1.5, 2, and 2.5µM) at 37°C under cell culture conditions. The number of viable leukemia cells was determined after 24, 48, and 72 hours. After 72 hours, VG-10 inhibited the growth of HL-60 cells with an IC_{50} value (IC_{50}= 50% growth inhibition of tumor cells) of 3.4µM, whereas VG-13 yielded an IC_{50} value of 5.5µM. VG-22 showed the lowest IC_{50} value and therefore was chosen for all further experiments. VG-22 inhibited the growth of HL-60 cells with an IC_{50} value (IC_{50}= 50% growth inhibition of tumor cells) of 2.3µM after 72 hours (table 1 & figures 8a-c).

Table 1: Biological activity of VG-10, VG-13, and VG-22 in HL-60 cells

		IC_{50} (µM)		
Agent	**MG**	**24h**	**48h**	**72h**
VG-10	356.44	7.3	3.9	3.4
VG-13	369.48	--	5.8	5.5
VG-22	314.79	--	--	2.3

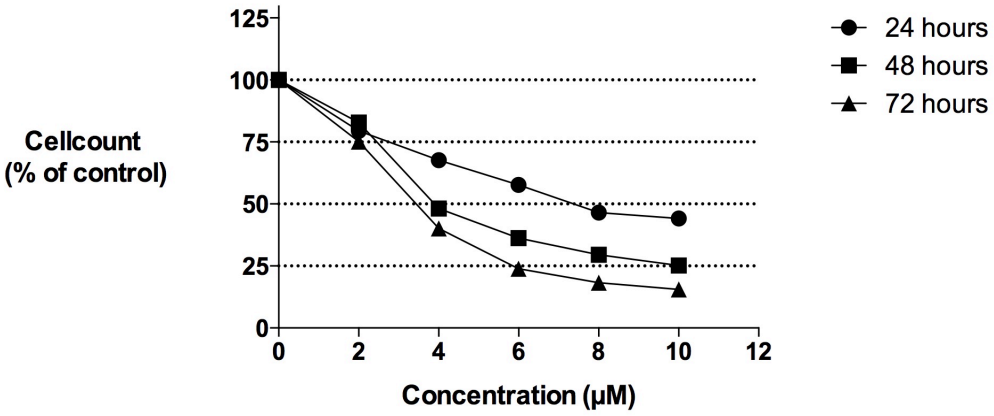

Figure 8a: Growth inhibition of HL-60 cells after incubation with VG-10

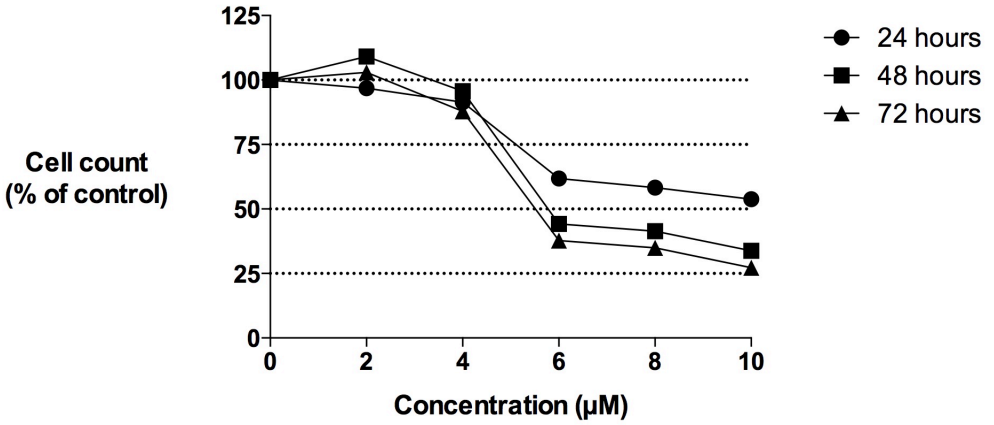

Figure 8b: Growth inhibition of HL-60 cells after incubation with VG-13

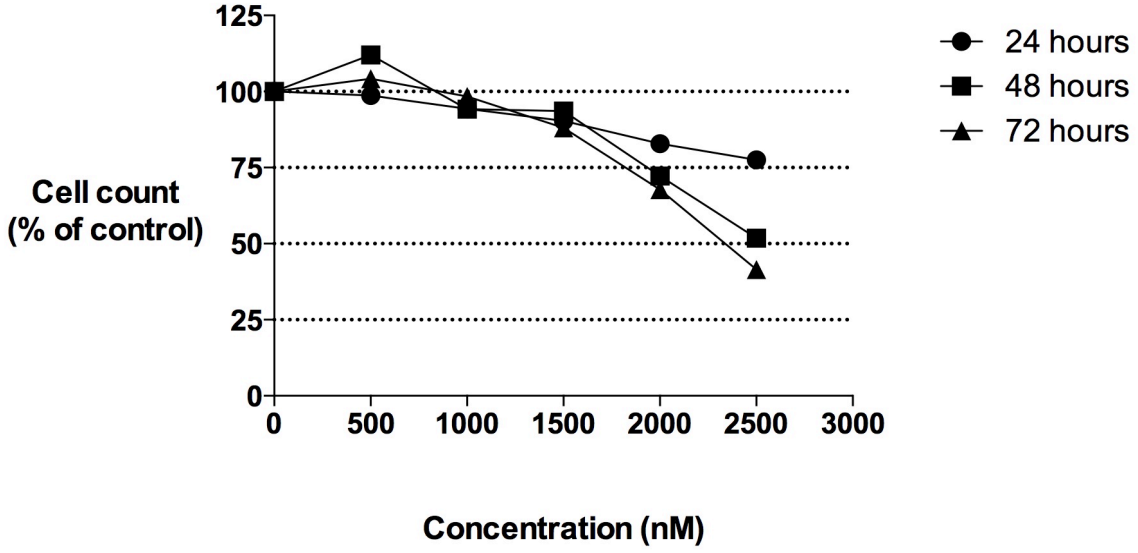

Figure 8c: Growth inhibition of HL-60 cells after incubation with VG-22

4.2. VG-22

4.2.1. Combination effects of VG-22 and arabinofuranosylcytosine (Ara-C) in HL-60 cells

To investigate the combination effects of VG-22 with Ara-C, HL-60 cells were cultivated at a concentration of 0.1x 10^6 per ml and simultaneously or sequentially incubated with increasing concentrations of VG-22 (1µM, 2µM, 3µM) and Ara-C (10nM, 15nM, 20nM). Three out of the nine drug combinations caused synergism after 48 hours when VG-22 and Ara-C were applied simultaneously and two out of nine drug combinations caused synergism after 72hours (table 2). When applied sequentially, five out of nine drug combinations yielded highly synergistic effects after 48 hours (table 3).

Table 2: Synergistic combination effects of VG-22 and Ara-C in HL-60 cells employing a simultaneous growth inhibition assay

		24h		48h		72h	
Compound	Concentration (µM/nM)	Cell number (% of control)	Combination Index*	Cell number (% of control)	Combination Index*	Cell number (% of control)	Combination Index*
VG22 (µM)	1	86.4		96.2		88.3	
	2	70.0		51.3		27.0	
	3	64.0		49.5		20.7	
AraC (nM)	10	65.1		72.3		66.1	
	15	56.8		61.1		53.7	
	20	50.4		54.9		42.5	
VG22 + AraC	1 10	63.4	**1.270**	51.6	**0.851***	40.2	**0.963**
VG22 + AraC	1 15	57.1	**1.297**	41.1	**0.811***	27.6	**0.882***
VG22 + AraC	1 20	49.9	**1.193**	33.5	**0.776***	18.0	**0.765***
VG22 + AraC	2 10	52.9	**1.047**	36.3	**0.909**	19.5	**0.951**
VG22 + AraC	2 15	47.4	**1.056**	35.2	**1.011**	18.9	**1.045**
VG22 + AraC	2 20	46.3	**1.215**	31.4	**1.030**	15.7	**1.040**
VG22 + AraC	3 10	47.4	**1.042**	37.5	**1.261**	18.6	**1.286**
VG22 + AraC	3 15	46.5	**1.218**	33.1	**1.275**	16.2	**1.304**
VG22 + AraC	3 20	45.4	**1.363**	28.8	**1.254**	13.2	**1.263**

Cells were simultaneously incubated with (1) VG-22 and (2) Ara-C for 24, 48, and 72h and the cell number was determined. Data is means of three determinations. Standard deviations (SD) were within 5%.

* Combination indices according to the equation of Chou and Talalay

Table 3: Synergistic combination effects of VG-22 and Ara-C in HL-60 cells employing a sequential growth inhibition assay.

		24 + 24h		24 + 48h		24 + 72h	
Compound	Concentration (µM/nM)	Cell number (% of control)	Combination Index*	Cell number (% of control)	Combination Index*	Cell number (% of control)	Combination Index*
VG22 (µM)	1	89.0		65.4		100.6	
	2	51.6		40.5		65.3	
	3	31.3		17.5		21.3	
AraC (nM)	10	63.8		61.7		82.9	
	15	57.2		56.7		76.5	
	20	54.4		54.0		70.9	
VG22 + AraC	1 10	50.4	**0.858***	50.0	**1.048**	74.6	**1.062**
VG22 + AraC	1 15	51.0	**1.079**	49.0	**1.167**	73.2	**1.297**
VG22 + AraC	1 20	46.1	**1.015**	41.4	**0.912**	57.0	**0.965**
VG22 + AraC	2 10	39.5	**0.969**	28.4	**0.902**	41.5	**0.942**
VG22 + AraC	2 15	37.7	**1.004**	23.6	**0.793***	31.8	**0.906**
VG22 + AraC	2 20	35.2	**0.996**	20.3	**0.717***	24.2	**0.864***
VG22 + AraC	3 10	27.9	**1.044**	13.2	**0.788***	11.5	**1.033**
VG22 + AraC	3 15	29.6	**1.126**	15.3	**0.865***	16.6	**1.112**
VG22 + AraC	3 20	26.9	**1.085**	11.4	**0.725***	8.8	**1.016**

Cells were sequentially incubated with (1) VG-22 for 24h and with (2) Ara-C for 24, 48, and 72h and the cell number was determined. Data are means of three determinations. Standard deviations (SD) were within 5%.

* Combination indices according to the equation of Chou and Talalay.

4.2.2. Fluctuations in dNTP pool sizes in HL-60 cells after incubation with VG-22 for 24 hours

Integral RR activity is responsible for equalized dNTP pools; therefore inhibiting the enzyme can cause pool size alterations and imbalances. To investigate this, a specific HPLC method (as previously described) was utilized.

HL-60 cells were incubated with 1µM, 2µM, and 3µM VG-22 for 24 hours. This caused alterations of intracellular dNTP pools with all three concentrations. Incubating cells with 1µM VG-22 resulted in a significant depletion of dCTP levels to 77.7%. dTTP and dATP levels were not significantly affected. Treatment with 2µM VG-22 resulted in a significant depletion of dCTP and dTTP concentrations to 78.2% and 69% respectively. Incubation with 3µM VG-22 decreased dCTP pool levels to 76%, dTTP pool levels to 81.2%, and dATP pool levels to 60.3%. dGTP pools were beyond the detection capability of the method (Figure 9).

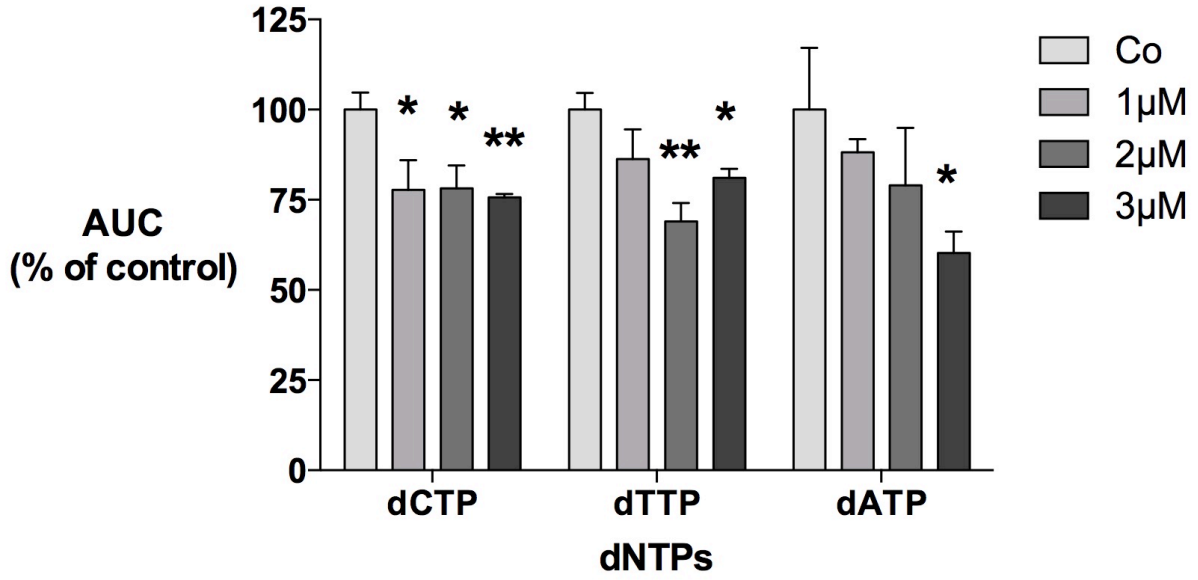

Figure 9: Concentration of dNTP pools in HL-60 cells after incubation with VG-22 for 24 hours

4.2.3. Expression of RR subunits R1, R2 and p53R2 after incubation with VG-22

HL-60 cells were incubated with 3µM VG-22 for 0.5, 2, 4, 8, and 24 hours in order to examine the influence of RR inhibitors on the expression of RR subunits and subsequently subjected to western blot analyses. The protein level of the constitutively expressed R1 subunit remained unchanged during the whole time course. The S-phase specific R2 subunit was elevated after two hours and the p53R2 subunit levels also remained unchanged after 24 hours of incubation (figure 10).

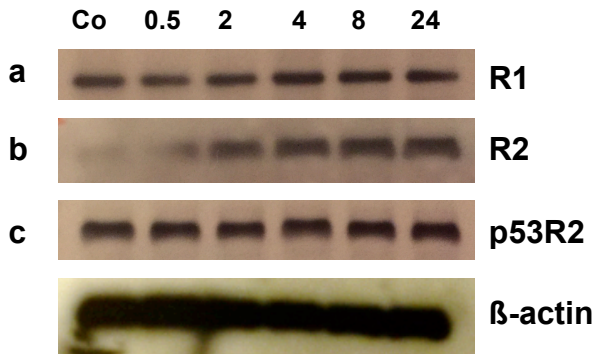

Figure 10: Expression levels of RR subunits in HL-60 cells after incubation with VG-22

4.2.4. Cell cycle distribution in HL-60 cells after incubation with VG-22 for 24 and 48 hours

HL-60 cells were incubated with 1µM, 2µM, and 3µM VG-22 for 24 and 48 hours or simultaneously incubated with VG-22 and Ara-C for 24 hours. Treatment of HL-60 cells with 1µM VG-22 for 24 hours did not cause a significant accumulation in S-phase, increasing the cell population from 38% to 40%. VG-22 concentrations of 2µM and 3µM led to a substantial cell population increase of 19% and 17%, respectively, and a G0-G1 phase cell decrease of 12% and 8%, respectively (figure 11a).
Treatment of HL-60 cells with 1µM and 2µM VG-22 for 48 hours caused no significant changes in cell cycle distribution. When treated with 3µM VG-22 for 48 hours, the cell population increased from 36% to 46% (figure 11b).
Simultaneous incubation of HL-60 cells with 2µM VG-22 and 15nM Ara-C led to a more obvious growth arrest in S-phase, increasing the cell population from 44% to 72%, while decreasing cells in G0-G1 phase from 43% to 17% (figure 11c).

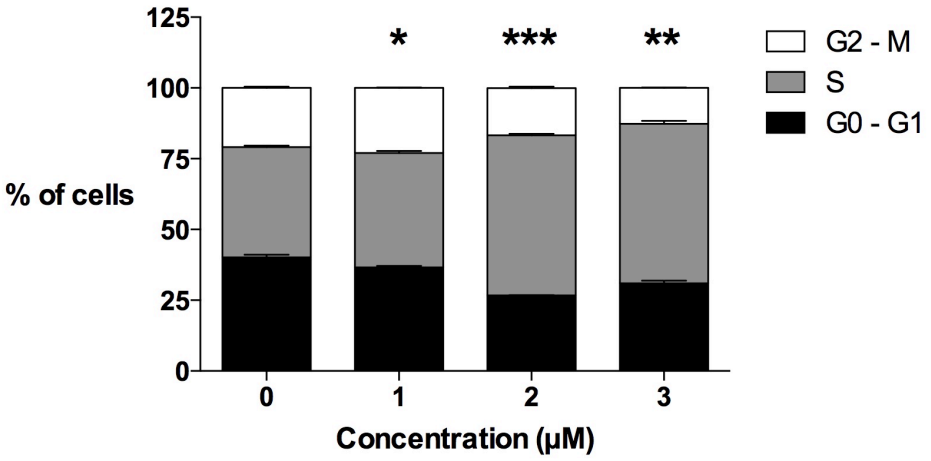

Figure 11a: HL-60 cell cycle distribution after incubation with VG-22 for 24 hours

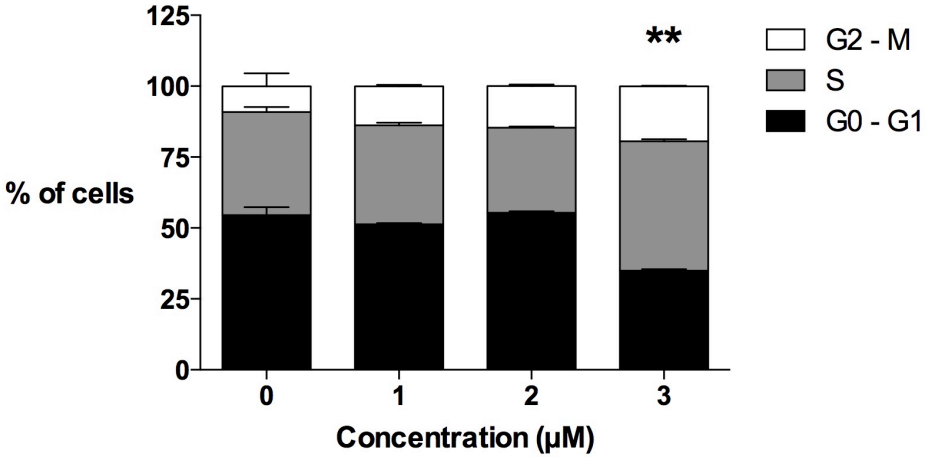

Figure 11b: HL-60 cell cycle distribution after incubation with VG-22 for 48 hours

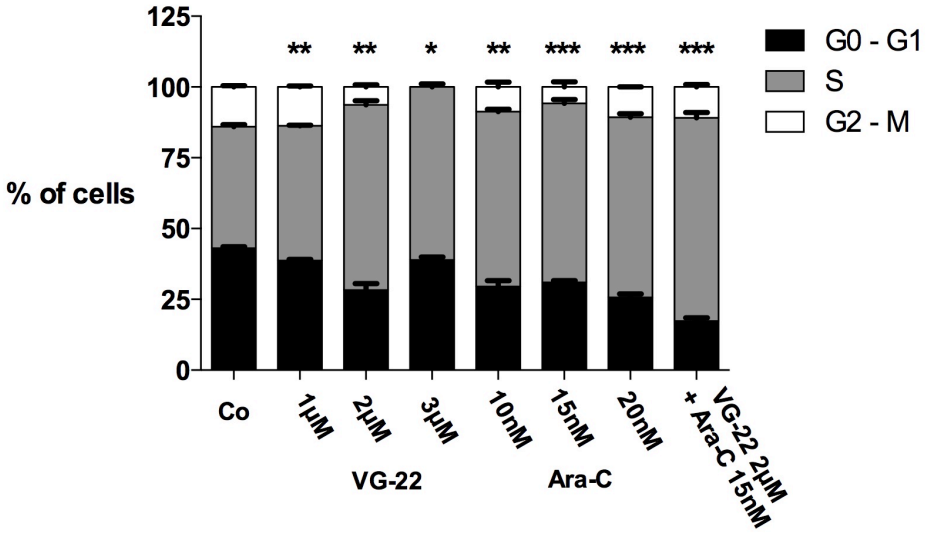

Figure 11c: HL-60 cell cycle distribution after simultaneous incubation with VG-22 and Ara-C for 24 hours

4.2.5. Expression of Chk1 kinase after incubation with VG-22

In order to investigate if S-phase inhibition caused an activation of cell cycle checkpoint kinases, HL-60 cells were incubated with 3µM VG-22 for 0.5, 2, 4, 8 and 24 hours and subsequently subjected to western blot analyses. Chk1 levels were elevated after 4 and 8 hours (figure 12).

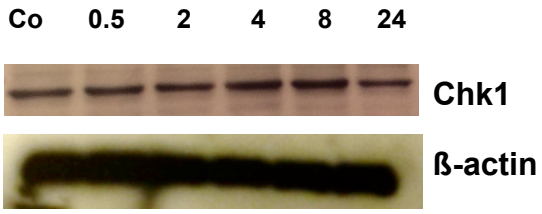

Figure 12: Expression of Chk1 in HL-60 cells after incubation with VG-22

5. Discussion

On the basis of the best QSAR model, 40 novel compounds with potential ribonucleotide reductase (RR) inhibiting properties were synthesized. RR is the rate-limiting enzyme for the *de novo* synthesis of deoxyribonucleotides (dNTPs). It is highly upregulated in tumor cells, as malignant tissues have an amplified need of deoxyribonucleoside triphosphates. Its inhibition could therefore be a breakthrough for cancer chemotherapy [4, 23].

Growth inhibition assays were conducted on all substances and those with the most promising IC_{50} values were chosen for more detailed testing. In a subsequent selection process, VG-22 was deemed the compound most active and therefore all further experiments were conducted with it.
After 72 hours, VG-22 inhibited the growth of HL-60 cells with an IC_{50} value of 2.3µM, thus demonstrating its cytotoxicity. Moreover, combination as well as synergistic effects were uncovered when VG-22 and Ara-C were applied simultaneously and sequentially, leading to an increased growth inhibition of HL-60 cells. Ara-C, a deoxycytidine antimetabolite, is a commonly used antitumor agent that inhibits DNA replication in leukemic cells. This suggests that the combination of VG-22 with existing antineoplastic drugs could enhance their properties [19, 20].

VG-22 also caused significant alterations and imbalances in dNTP pool size, suggesting an influence on the integral RR activity. Especially when applied with concentrations of 2µM and 3µM, significant depletions in dCTP and dTTP levels were obtained. This indicates that *in situ* RR activity is inhibited and through this imbalance, *de novo* DNA synthesis is faulted. Subsequently, cell proliferation cannot take place as usual.

The protein levels of the R1 subunit of RR as well as the p53R2 subunit remained unchanged, while the S-phase specific R2 subunit was elevated after two hours of incubation. The upregulation of the R2 subunit protein levels can be argued as a response to the DNA damage and usually also involves an upregulation and activation of Chk1 [4]. However, the almost complete absence of R2 in the control of the west-

ern blot indicates that the results might not be accurate. Further testing should be undertaken to clarify the expression levels. Unaltered levels of R1 subunit and S-phase specific R2 subunit would be coherent with the fact that the enzyme activity can be arrested without influencing the protein levels of its subunits [5].

Cell cycle distribution results revealed a strong accumulation in S-phase when HL-60 cells were treated with VG-22 for 24 hours. After 48 hours, however, S-phase accumulation only took place when HL-60 cells were treated with 3μM VG-22, which could suggest that VG-22 causes transient cell cycle arrest, as opposed to apoptosis. Simultaneous incubation of HL-60 cells with VG-22 and Ara-C resulted in a very significant growth arrest in S-phase. This shows that the combination of the two agents caused a highly synergistic effect, which goes in accordance with the findings of the growth inhibition assays conducted. Furthermore, the significant S-phase arrest is coherent with the fact that RR is a rate-limiting enzyme for S-phase transition. When RR is inhibited, cells enter S-phase at a normal rate, yet accumulate there and eventually undergo apoptosis [4].

Cell cycle checkpoint kinases are activated by DNA damage, incomplete DNA replication or an imbalance of dNTP pools in order to halt DNA synthesis and allow time for repair before damage is passed on to daughter cells. Before undergoing mitosis, cells have to pass several cell cycle checkpoints. These are controlled by specific key regulator proteins, ATR and ATM kinases. Downstream effector kinase Chk1 is activated by ATR, whereas Chk2 is activated by ATM [4].
Chk1 levels were elevated after 4 and 8 hours demonstrating the cell's response to the DNA damage.

In conclusion, it can be said that VG-22 represents a promising RR inhibitor that provides antineoplastic effects especially in combination with the known antitumor drug Ara-C. Therefore it deserves further *in vitro* as well as *in vivo* testing.

6. Summary

As cancer is the second leading cause of death worldwide, it becomes clear that efforts on finding and developing new antineoplastic agents are a priority in today's world. New synthetic agents with possibly fewer side effects are now being sought after more than ever.

The rate-limiting enzyme ribonucleotide reductase (RR) is responsible the *de novo* synthesis of deoxyribonucleotides (dNTPs). It is found in all growing cells and is especially upregulated in malignant tissues, since these have an amplified need of deoxyribonucleoside triphosphates. Therefore RR is considered an exceptional target for cancer chemotherapy.

In this diploma thesis, a novel group of thiosemicarbazones (VG-series) was investigated for their anticancer activity in the human HL-60 promyelocytic leukemia cell line. VG-22, the most promising compound out of the series, was designed on the basis of the best QSAR model and reviewed in depth for its cytotoxicity. It was tested on its ability to inhibit RR activity, thus arresting DNA replication, its ability to induce apoptosis and its capability to cause general cell growth arrest.

Effectively, VG-22 proved to be a powerful RR inhibitor. It instigated alterations in dNTP pool sizes and caused leukemia cells to accumulate in S-phase of the cell cycle. It therefore led to a transient cell cycle arrest rather than apoptosis and activated cell cycle checkpoint kinase Chk1.
Furthermore analyses showed, that when VG-22 was combined with the well-known and established antitumor agent Ara-C, potentiation of the antineoplastic effects could be denoted.

Based on these results, VG-22 may possibly be a candidate for further research in *in vitro* as well as *in vivo* testing.

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7. Zusammenfassung

Da Krebs die zweithäufigste Todesursache weltweit darstellt, ist es ersichtlich, dass die Suche nach bzw. die Entwicklung neuer antineoplastischer Mittel eine Priorität in der heutigen Gesellschaft ist. Das Ziel ist es neue synthetische Wirkstoffe, mit möglicherweise weniger Nebenwirkungen zu finden.

Die Ribonucleotidreduktase (RR) ist ein Enzym, welches für die de novo Synthese von Deoxyribonucleotiden (dNTPs) verantwortlich ist. Es kommt ubiquitär in allen wachsenden Zellen vor, und ist besonders in malignen Geweben hochreguliert, da diese einen erhöhten Bedarf an Deoxyribonukleosidtriphosphaten haben. Es stellt dadurch ein interessantes und geeignetes Angriffsziel für chemotherapeutische Behandlungen dar.

Im Zuge dieser Diplomarbeit wurde eine neue Gruppe von Thiosemicarbazone (VG-Serie) auf ihre antineoplastischen Fähigkeiten in humanen HL-60 Promyelozytenleukämiezellen untersucht. VG-22, die vielversprechendste Verbindung aus der Serie, wurde basierend auf das beste QSAR Modell entwickelt und genauer in Bezug auf ihre Zytotoxizität untersucht. Sie wurde auf ihre Fähigkeit RR zu inhibieren, einen Zellwachstumsstillstand auszulösen sowie ihre Fähigkeit Apoptose zu induzieren untersucht.

Tatsächlich erwies sich VG-22 als RR Inhibitor. Sie verursacht eindeutige Veränderungen der Konzentrationen der dNTP Pools und bewirkt außerdem eine Akkumulation von Leukämiezellen in der S-phase des Zellzyklus. Es kam zu einem transienten Zellzyklusarrest und nicht zur Apoptose und Zellzyklus Checkpoint Kinase Chk1 wurde aktiviert.
Weiters wurde festgestellt, dass VG-22 in Kombination mit dem etablierten Antitumormittel Ara-C synergistisch wirkt bzw. eine Potenzierung der antineoplastischen Wirkung bezeichnet werden konnte.

Basierend auf diesen Ergebnissen repräsentiert VG-22 möglicherweise einen Kandidaten sowohl für weitere in vitro als auch für in vivo Untersuchungen.

Table of Figures

List of Tables

8. References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

2. American.Cancer.Society. 2015. Available from: http://www.cancer.org.

3. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod. 2007;70(3):461-77.

4. Saiko P, Graser G, Giessrigl B, Lackner A, Grusch M, Krupitza G, et al. A novel N-hydroxy-N'-aminoguanidine derivative inhibits ribonucleotide reductase activity: Effects in human HL-60 promyelocytic leukemia cells and synergism with arabinofuranosylcytosine (Ara-C). Biochem Pharmacol. 2011;81(1):50-9.

5. Saiko P, Steinmann MT, Schuster H, Graser G, Bressler S, Giessrigl B, et al. Epigallocatechin gallate, ellagic acid, and rosmarinic acid perturb dNTP pools and inhibit de novo DNA synthesis and proliferation of human HL-60 promyelocytic leukemia cells: Synergism with arabinofuranosylcytosine. Phytomedicine. 2015;22(1):213-22.

6. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.

7. Alberts B. Molecular biology of the cell. 5th ed. ed. New York: Garland Science ; [London : Taylor & Francis, distributor]; 2008: 1053-1114.

8. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 2003;36(3):131-49.

9. BD.Biosciences. 2015. Available from: http://www.bdbiosciences.com/br/research/apoptosis/analysis/index.jsp.

10. Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Prolif. 2003;36(3):165-75.

11. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ. 2009;16(1):3-11.

12. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta. 2013;1833(12):3448-59.

13. National.Cancer.Institute. 2015. Available from: http://www.cancer.gov.

14. Guzman ML, Jordan CT. Considerations for targeting malignant stem cells in leukemia. Cancer Control. 2004;11(2):97-104.

15. Krause DS, Van Etten RA. Right on target: eradicating leukemic stem cells. Trends Mol Med. 2007;13(11):470-81.

16. Misaghian N, Ligresti G, Steelman LS, Bertrand FE, Basecke J, Libra M, et al. Targeting the leukemic stem cell: the Holy Grail of leukemia therapy. Leukemia. 2009;23(1):25-42.

17. Richard A. Larson M. Remission criteria in acute myeloid leukemia and monitoring for residual disease 2015. Available from: http://www.uptodate.com/contents/remission-criteria-in-acute-myeloid-leukemia-and-monitoring-for-residual-disease.

18. Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. Cancer. 2006;107(9):2099-107.

19. Estey E, Dohner H. Acute myeloid leukaemia. Lancet. 2006;368(9550):1894-907.

20. Momparler RL. Optimization of cytarabine (ARA-C) therapy for acute myeloid leukemia. Exp Hematol Oncol. 2013;2:20.

21. Appelbaum FR, Rowe JM, Radich J, Dick JE. Acute myeloid leukemia. Hematology Am Soc Hematol Educ Program. 2001:62-86.

22. MedicineNet I. 2012. Available from: http://www.medicinenet.com/script/main/art.asp?articlekey=19758.

23. Cerqueira NM, Fernandes PA, Ramos MJ. Ribonucleotide reductase: a critical enzyme for cancer chemotherapy and antiviral agents. Recent Pat Anticancer Drug Discov. 2007;2(1):11-29.

24. Hochtl T, Horvath Z, Bauer W, Karl D, Saiko P, Elford HL, et al. Biochemical modulation of Ara-C effects by amidox, an inhibitor of ribonucleotide reductase in HL-60 promyelocytic human leukemia cells. Life Sci. 2004;74(9):1071-80.

25. Kolberg M, Strand KR, Graff P, Andersson KK. Structure, function, and mechanism of ribonucleotide reductases. Biochim Biophys Acta. 2004;1699(1-2):1-34.

26. Reichard P. Ribonucleotide reductases: substrate specificity by allostery. Biochem Biophys Res Commun. 2010;396(1):19-23.

27. Zhou B, Shao J, Su L, Yuan YC, Qi C, Shih J, et al. A dityrosyl-diiron radical cofactor center is essential for human ribonucleotide reductases. Mol Cancer Ther. 2005;4(12):1830-6.

28. Wijerathna SR, Ahmad MF, Xu H, Fairman JW, Zhang A, Kaushal PS, et al. Targeting the Large Subunit of Human Ribonucleotide Reductase for Cancer Chemotherapy. Pharmaceuticals (Basel). 2011;4(10):1328-54.

29. Aye Y, Li M, Long MJ, Weiss RS. Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. Oncogene. 2015;34(16):2011-21.

30. Saiko P, Pemberger M, Horvath Z, Savinc I, Grusch M, Handler N, et al. Novel resveratrol analogs induce apoptosis and cause cell cycle arrest in HT29 human

colon cancer cells: inhibition of ribonucleotide reductase activity. Oncol Rep. 2008;19(6):1621-6.

31. Baldini M, Belicchi-Ferrari M, Bisceglie F, Dall'aglio PP, Pelosi G, Pinelli S, et al. Copper(II) complexes with substituted thiosemicarbazones of alpha-ketoglutaric acid: synthesis, X-ray structures, DNA binding studies, and nuclease and biological activity. Inorg Chem. 2004;43(22):7170-9.

32. Opletalova V, Kalinowski DS, Vejsova M, Kunes J, Pour M, Jampilek J, et al. Identification and characterization of thiosemicarbazones with antifungal and antitumor effects: cellular iron chelation mediating cytotoxic activity. Chem Res Toxicol. 2008;21(9):1878-89.

33. Paterson BM, Donnelly PS. Copper complexes of bis(thiosemicarbazones): from chemotherapeutics to diagnostic and therapeutic radiopharmaceuticals. Chem Soc Rev. 2011;40(5):3005-18.

34. Selleck.Chemicals. 2013. Available from: http://www.selleckchem.com/products/triapine.html.

35. Berger W, Heffeter P. Thiosemicarbazones 2015. Available from: http://krebsforschung.meduniwien.ac.at/forschung-research/research-focus/applied-and-experimental-oncology/walter-berger-univ-prof-dr/petra-heffeter-univ-ass-mag-dr/thiosemicarbazones/

36. Serda M, Kalinowski DS, Rasko N, Potuckova E, Mrozek-Wilczkiewicz A, Musiol R, et al. Exploring the anti-cancer activity of novel thiosemicarbazones generated through the combination of retro-fragments: dissection of critical structure-activity relationships. PLoS One. 2014;9(10):e110291.

37. Schönhuber A. Effects of small molecules on human leukemic cells: Combination therapy with arabinofuranosylcytosine (Ara-C). Uniwien, 2015

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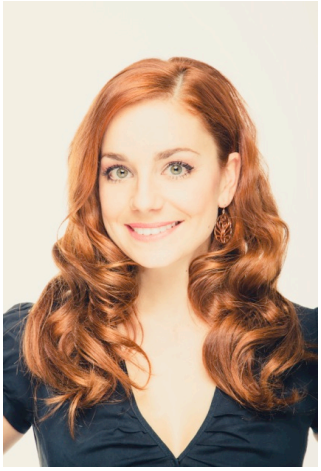
'Gratitude is not only the greatest of virtues, but the parent of all the others.' -Cicero

10. Curriculum Vitae



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