universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Effects of small molecules on human leukemic cells:

Combination therapy with arabinofuranosylcytosine (AraC)“

verfasst von / submitted by

Agnes Sophie Schönhuber

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2015 / Vienna, 2015

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Ao. Univ.Prof. Mag.pharm. Dr. Walter Jäger

Table of contents

List of tables

List of figures

Abbreviation Index

ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATP	adenosine triphosphate
AraC	Arabinofuranosylcytosine
ATCC	American type culture collection
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
dCK	Deoxycytidine kinase
dFdC	Gemcitabine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
G0, G1, G2	gap phases of the cell cycle
HL-60	acute promyelocytic leukemia cell line
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HU	Hydroxyurea
IC50	half maximal inhibitory concentration
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
RNA	ribonucleic acid
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 aims

Regarding to 14 million new cases of cancer in 2012, the disease is the leading reason of death worldwide. More than 100 different types of cancer exist. Although a multitude of treatments has been developed so far, the number of new cancer cases will rise to 22 million within the next two decades. Thus, there exists an urgent need for the discovery and development of novel compounds [1].

There are many types of cancer treatments like surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy or stem cell transplant. Most people have a combination of treatments, such as surgery with chemotherapy and/or radiation therapy. Despite this various possibilities, cytotoxic chemotherapy associated with relatively high toxicity remained the only option in the treatment of advanced or metastatic cancer over the past few decades. However, new technologies enable synthesis and drug screening of novel compounds which appear to be very promising in their antineoplastic effect.

The Department of Pharmaceutical Sciences, Birla Institute, Mestra, India, synthesized and provided a novel group of thiosemicarbazones (VG-series), all of which were supposed to have an antineoplastic effect on cells by inhibiting the enzyme ribonucleotide reductase (RR). RR reduces ribonucleoside diphosphates into deoxyribonucleoside diphosphates, which are the building blocks for DNA *de novo* synthesis and therefore essential for DNA replication. In addition, RR is the rate-limiting enzyme by maintaining a balanced pool of all four deoxyribonucleoside triphosphate (dNTPs) in the cell. Thus, RR is responsible for the cell proliferation. In cancer cells, RR is highly upregulated because of their increased need of DNA. As a consequence, the enzyme is expected to be a sensitive target for cancer chemotherapy [2].

In this diploma thesis, these novel compounds were investigated for their anticancer activity in the human HL-60 promyelocytic leukemia cell line. Different growth inhibiting assays allowed to select the most promising compound regarding to their IC_{50}. Additionally, the combination effects of these agents with AraC, a well-established anticancer drug, was examined. Therefore, agents were applied simultaneously or sequentially. Moreover, cell cycle effects were evaluated by FACS.

Radiolabeled cytidine was incorporated into DNA of tumor cells to determine the *in situ* activity of RR. In addition, in order to determine the equilibrium of dNTP pools, a specific HPLC method was conducted. Western blot analyses displayed a better insight into the mechanisms of apoptotic and growth inhibiting pathways.

A high number of novel drugs are in the pipeline awaiting clinical development. It will be essential to synthesize new therapeutic agents to create more effective cytotoxics by combining them [3].

In this diploma thesis, very promising results were obtained and VG-12 as single drug as well as in combination with AraC might contribute to the clinical establishment of new therapies of human malignancies.

2. Introduction

2.1. Cancer and its generation

Cancer is a generic term for a worldwide complex group of diseases involving excessive cell growth. There exists a variety of about 100 different types of cancer located on diverse organs. One defining in cell cycle progression, leads to unregulated cell proliferation in an uncontrolled manner. Proto-oncogenes are involved in normal cell growth and division. The gain-of-function mutations generate proto-oncogenes which allow them to grow out of control and invasive and let them survive when they should not. In addition, DNA repair genes are involved in fixing damaged DNA. Cells with mutations in these genes tend to develop additional mutations in other genes. Together, these mutations may cause the cells to become cancerous. In addition, damaged DNA can be fxed by so-called DNA repair genes. If this genes are mutated, they show a tendency to develop additional mutation in other genes. Altogether, those may be the reasons why cells cecome cancerous [4].

The hallmarks of cancer

It is suggested that the genomes of cancer types undergo six fundamental cellular alterations that impact their cell physiology and led them result in malign cell growth. As shown in Fig. 1, these so-called hallmarks of cancer include self-sufficiency in growth signals, evasion of apoptosis, activation of tissue invasion and metastasis, indifference to growth inhibitory signals, sustained angiogenesis and permanent replicative potential. These alterations can appear in one point mutations as well as in several mutations in chromosome complement. Not only in combination but also each mutation by itself provides cancer cells an advantage over normal cells and allows them to develop faster and to invade surrounding tissues [5].

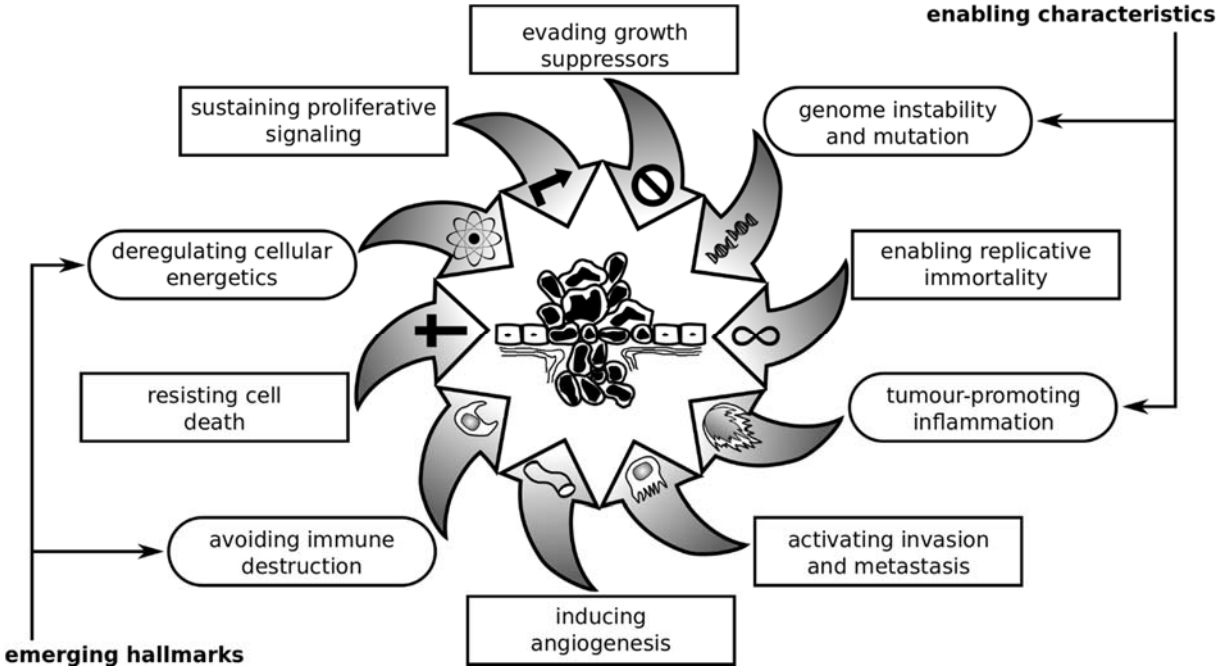


Fig. 1: Fundamental properties in cell physiology causing cancer [6]

Supplementary to these six hallmarks of cancer, the same authors recently proposed two new hallmarks that could possibly play a role in the cancerogenesis: One involves the ability of cancer cells to alter, thus modulating a cells' metabolism. Consequently, this mechanism could support degenerated proliferation. The other hallmark pertains the avoidance of immune destruction. Thereby cancer cells are able to escape extinction by leucocytes or macrophages. Due to the fact that both capabilities are not fully approved, they are so-called emerging hallmarks [5].

2.2 Cell cycle and Cancer

2.2.1 Cell division cycle

The cell division cycle is a complexe process in order to divide and to duplicate the cell. As a final result, two daughter cells are produced. The cycle can be divided into two basic parts: mitosis and interphase. During mitosis, daughter chromosomes get separated. The phase ends with cell division, so called cytokinesis. Most time of the cell cycle is spend in the period between mitoses, named interphase. In order to prepar the DNA for cell division, during interphase the chromosomes are decondensed and distributed which leads to a morphologically uniform nucleus. At the molecular level, interphase is divided in four phases: M phase, G_1 phase, S phase and G_2 phase. M phase corresponds to mitosis; during G_1 phase (gap 1), the cell grows but does not yet duplicate its DNA. After, S phase takes place in order to synthetise DNA. G_2 phase is following and allows to complete the duplication by continuous growth and synthesis of proteins (Fig. 2) [7].

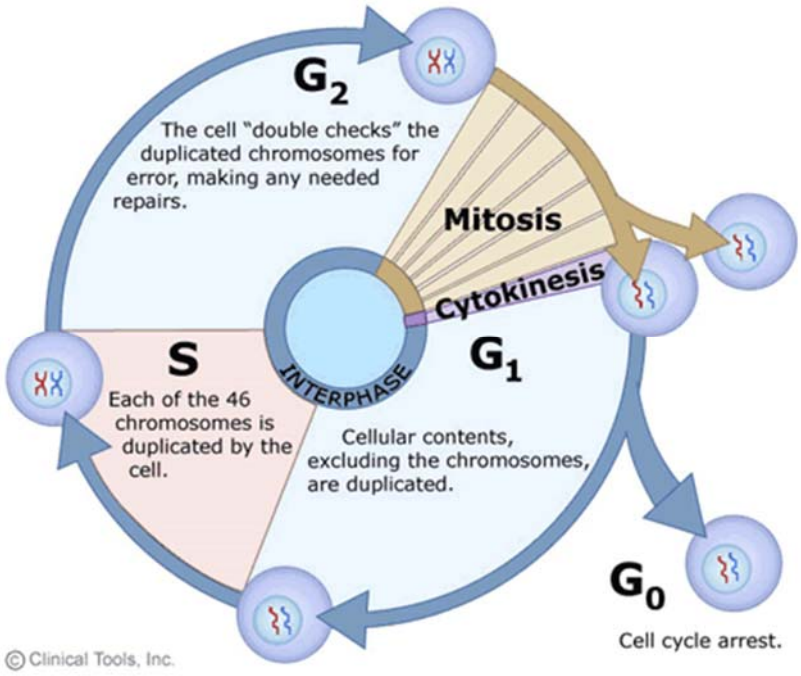


Fig. 2: Cell division cycle [8]

1.2.2 Cell cycle regulation and control

As already mentioned, the cell division cycle is a complexe process. To guarantee a highly-ordered process of cell division cycle progression, multiple checkpoints are able to assess extracellular growth signals, cell size and DNA integrity. Two protein kinases, called ATR and ATM, are able to control the activation of the cell cycle checkpoint kinases [9].

In case of need, the cell cycle may be arrested at the G1, intra-S or G2 checkpoints. At this point, ATR and ATM protein kinases function as key regulator by activating their downstream effector kinases, called Chk1 and Chk2 (Fig. 3) [10, 11].

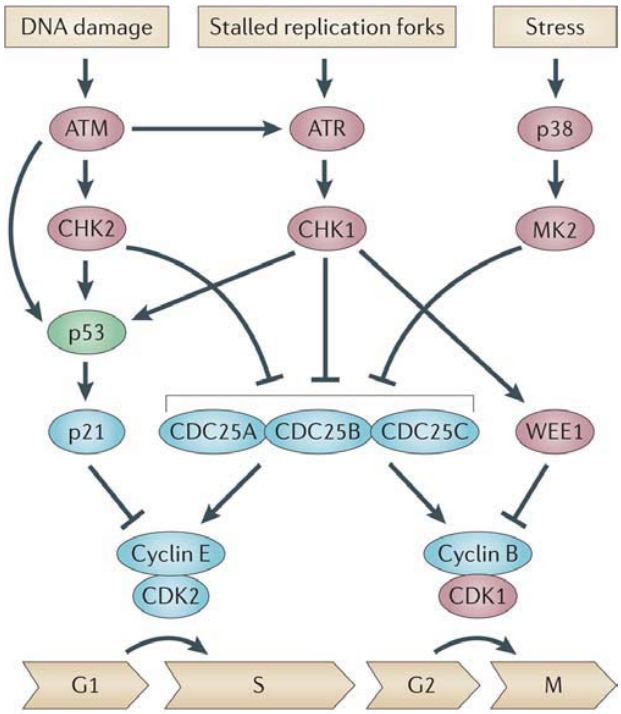


Fig. 3 DNA damage-induced cell cycle checkpoints [11]

The checkpoints are put together of a group of enzymes, so called cyclin-dependent kinases (CDKs). Together with at least two proteins (depending on the type of cyclin), a kinase and a cyclin, the CDK becomes activated (Tab. 1). CDKs and their cyclin partner are positive regulators or accelerators that induce cell cycle progression [10].

Tab. 1: Mammalian cyclin-dependent kinases (CDKs) and their regulatory Cyclins

CDKs	Cyclins
Cdc2	Cyclin A & B
Cdk2	Cyclin A ,E & D
Cdk3	Cyclin E
Cdk4	Cyclin D1, D2, D3
Cdk5	p35
Cdk6	Cyclin D1, D2, D3
Cdk7	Cyclin H
Cdk8	Cyclin C

Different cyclin proteins are needed in different stages of the cell cycle. As the cells progress from G1 to mitosis, cyclin D, E, A, and B are expressed with CDK4, CDK6, CDK2, and CDC2, respectively. Cyclin D activates CDK4 or CDK6 in G0/G1 phase, whereas cyclin E or A forms a complex with CDK2 in G1/2 phase and during phase G2 cyclin A or B is associated with CDC2 (Fig. 4) [10].

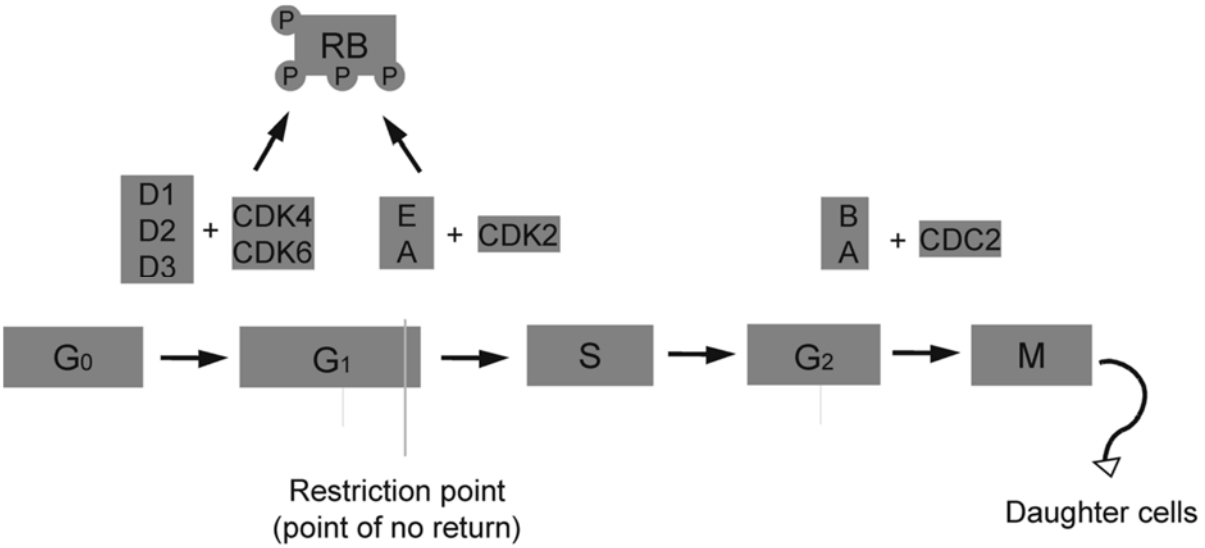


Fig. 4: Progression of cell cycle [12]

2.2.2 Cell cycle deregulation in cancer

Cancer cells differ from normal cells in many characteristics. The molecular analysis of human tumors has shown that cell cycle regulators are frequently mutated in human tumors: mutations in the expression of various cyclins, CDKs and CKIs are frequently associated with tumor genesis. Thus, cyclins might be directly involved in the development of human malignancies. This breakdown in the accurate regulation of the cell cycle can bring about serious consequences [9].

2.2.3 Cell death

As already mentioned, dying cells are involved in a process that is reversible until a first irreversible phase or point-of-no-return is passed. Due to the missing clearly defined biochemical event that could be considered as the point-of-no-return, the Nomenclature Committee on Cell Death (NCCD) proposes following criterias to consider a cell as dead [13]: the cell lost the integritiy of its plasma membrane; the cell as a whole is fragmented into discrete bodies; and/or its corpse are swallowed up by another cell *in vivo* [7].

Apoptosis

Apoptosis is characterised by following morphological alterations: retraction of pseudopods, reduction of cellular volume, chromatin condensation, nuclear fragmentation, little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing or being swalloed by phagocytes (*in vivo*) [7]. Apoptosis is a gene-directed program, thus the cell numbers can be regulated by factors that influence cell survival as well as proliferation and differentiation. Therefore, apoptosis intends to protect against the development of cancer [14]. A cascade of events starts when the apoptotic regulatory system is activated, resulting in the proteolysis of the cell. In this process, different proteins affect either an inhibition (e.g. Bcl-2) or an initiation (e.g. Bax and Bak) of apoptosis. By binding to Bax and Bak, Bcl-2 is able to supress apoptosis. Otherwise, Bax and Bak disrupts the outer mitochondrial membrane integrity, which results in the release of proapoptotic signalling proteins [5].

Faults in apoptotic pathways can be the reason for a diversity of human diseases, varying from neurodegenerative disorders to malignancy. The expression of apoptosis inhibitors is increased in tumor cells, whereas the expression of apoptotic initiators is down

regulated. Moreover, in many cases the tumor suppressor protein p53 loses its function, thus the ability to initiate the programmed cell death is lost and tumor cells are able to evade apoptosis [1, 14].

Necrosis

The term necrosis should be used by the appereance of following morphologically features: gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents in the local tissue [13]. Compared to apoptotic cell death, no caspases are involved in necrotic cell death and inflammatory reactions are usually included. Inflammatory cells are recruited to examine the damage and to remove the necrotic remains. It is suggested that they may promote tumors because of their ability to promote angiogenesis and cell proliferation [15].

Autophagic cell death

Autophagic cell death is morphologically defined as a type of cell death accompanied by massive autophagic vacuolization of the cytoplasm, thus being considered a self-cannibalization method. In contrast to apoptotic cells, dying cells have little or no association with phagocytes. Autophagic cell death plays an important role in maintaining healthy mammalian cells from nutrient stress, aggregates of misfolded proteins, organelle damage and microbes and is essential for survival [16]. Despite the cytoprotective effect to the organism, exuberant autophagy may be very damaging and can promote cell death in healthy cells as well as in tumor cells [5, 15].

2.3 Ribonucleotide reductase

Deoxyribonucleic acid (DNA) stores the biological information in all cellular organisms and many viruses. It is built of deoxyribonucleotides (dNTP), which are synthesized by reduction of ribonucleotides, which are the building blocks of RNA.

Ribonucleotide reductase (RR) is an enzyme that catalyses the conversion of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates, hence it is an essential enzyme for the *de novo* DNA synthesis and DNA repair [17].

2.3.1 Function and structure

Accurate DNA replication is essential for proper development, growth and tumor-free survival in all multicellular organisms. Therefore, RR is the rate-limiting enzyme by means of maintaining a balanced pool of all four dNTPs in the cell.

By transforming the RNA building blocks into DNA building blocks, protein-derived cysteinyl free radicals are generated by three profoundly different mechanisms. This radicals are supposed to be part of the mechanism that abstracts the hydrogen atom on the ribose ring of the RNA. Due to the way the radicals are generated, RR is divided into three homologous classes [18].

Class I enzymes are found in all eukaryotic organisms and some prokaryotes. They all depend on oxygen and can be further divided into two subgroups: Ia and Ib. The human RR belongs to Class Ia and consists of a large α_2- homodimer (R1) and of a small β_2-homodimer (R2).

R1 contains the allosteric and the catalytic sites and takes part in the reduction of the substrate, R2 houses an diferric-tyrosyl radical factor that is required to initiate nucleotide reduction in R1 [19].

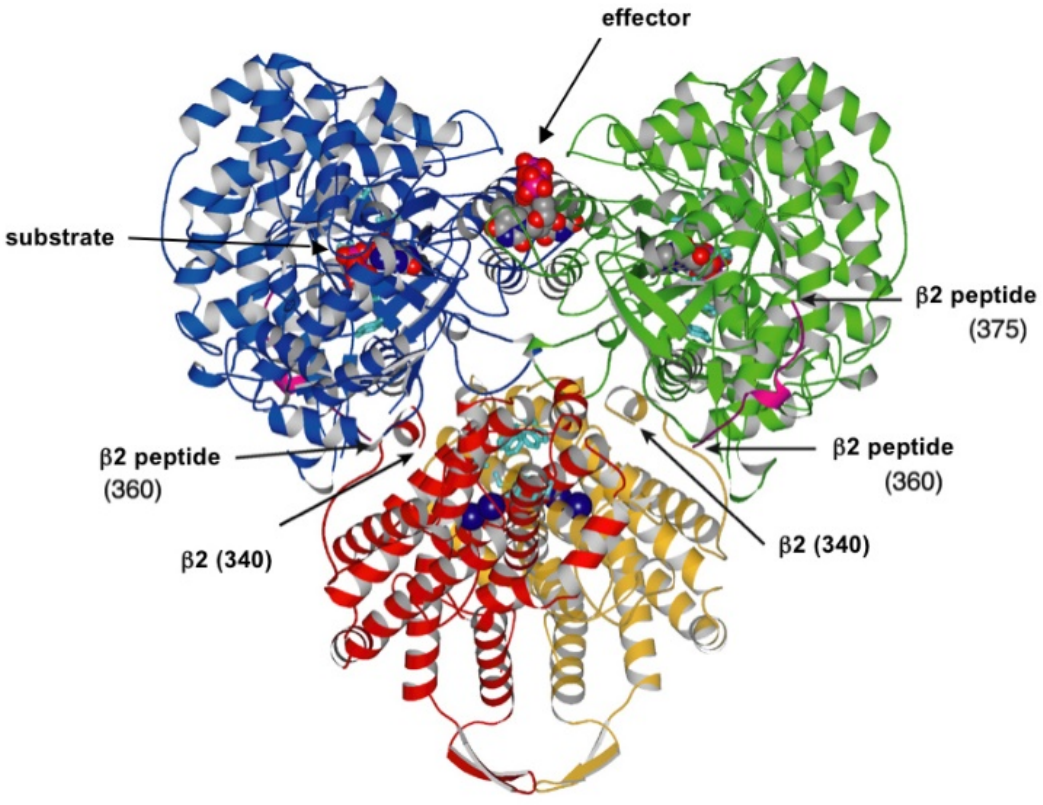


Fig. 5: Class I ribonucleotide reductase [20]

2.3.2 Regulation

RR is able to reduce all four ribonucleotides [21]. In order to provide a balanced supply of dNTPs for DNA replication, the enzyme is regulated by a difficult allosteric regulation. Corresponding to the allosteric configuration, one of the four ribonucleotides can bind to the active side [22, 23, 24]

The process of reduction is tightly regulated via two allosteric sites located in the R1 subunit, the specificity site (s-site) and the overall activity side (a-site). The a-site resides in an N-terminal ATP cone domain that binds dATP or ATP and functions as an on/off switch, whereas the composite s-site binds ATP, dATP, dTTP, or dGTP and determines which substrate will be reduced. Thus, when ATP binds at the allosteric site, RR gets activated. Otherwise, binding of dATP at this site deactivates RR. (Fig. 6) Therefore, the control of RR is important not only because of its influence of the kinetics of DNA replication but also because of its effect of the integrity of the genome [25].

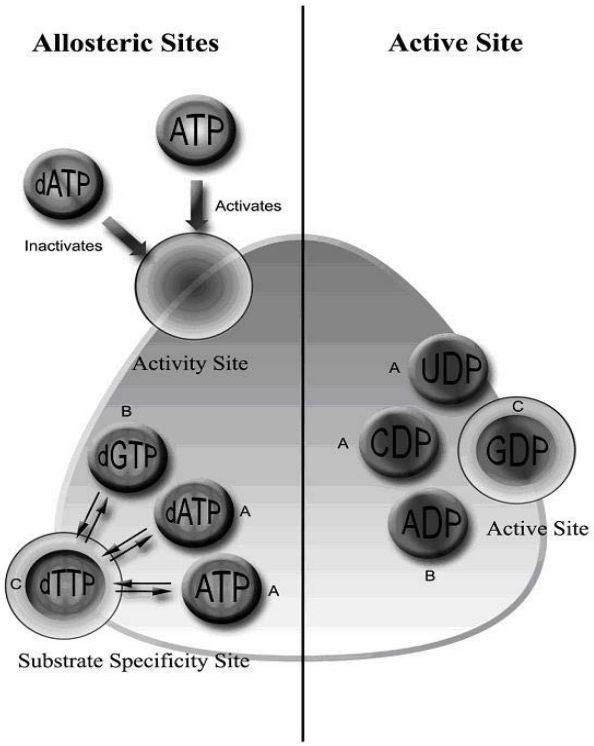


Fig. 6: Regulation of class I RRs. Class I RRs are activated by binding ATP or inactivated by binding dATP to the activity site located on the RR1 subunit [26].

2.3.3. RR inhibitors and cancer

As already mentioned, RR is responsible for the appropriate supply of all four dNTPs, which are essential for DNA replication and DNA repair [27]. Due to the increased proliferation rate of cancer cells in comparison to native cells, they need large amounts of dNTPs. Because of this dependency on the availability of deoxyribonucleotides, RR is an sensitive target to treat cancer cells [27]. Inhibition of RR leads to disparities and alterations in dNTP pool levels [28].

Clinically established RR inhibitors are hydroxyurea (HU), fludarabine, diflurodeoxycytdine (Gemcitabine, dFdC), and Cytarabine (AraC).

Both AraC and dFdC first are modified by deoxycytidine kinase in such way, that RR spuriously recognises them as natural nucleosides. AraC acts as a substrat of deoxycytidine kinase (dCK) and is metabolized amongst others to the antimetabolite AraC-TP. The main cytotoxic effect of AraC-TP is the inhibition of the DNA-polymerase [29]. In addition, interaction with the active side of R1 leads to deficient products that inactivate RR [30].

HU is able to destroy the tyrosyl radical, which stabilizes the dinuclear iron center of the R2 subunit. As a result, the enzyme as a whole is destabilized and loses its function [31].

2.4 Leukemia

2.4.1 Definition and categorization

Leukemia stand for a group of cancers that originate in the bone marrow, where most blood cells are formed by hematopoietic stem cells. These cells are able to renew themselves, proliferate and differentiate into different cell types.

Hence, mutations that induce uncontrolled growth or attenuate cell development result in high numbers of abnormal, not fully developed white blood cells, which enter the bloodstream. These cells are called blasts or leukemia cells.

Depending on the type of blood cell that has become cancerous (lymphoid versus myeloid) and on the progress of disease (acute versus chronic), the leukemias can be categorized: Acute lymphoid leukemia (ALL) is characterized by overproduction and accumulation of lymphoblasts (immature white blood cells). Hence, it inhibits the production and

development of other cells in the bone narrow and by immigrating into the bloodstream and into other organs. Also, lymphoid leukemia can appear in a chronic way (CLL).

Acute myeloid leukemia (AML) is a cancer of myeloid cells (immature white blood cells that are not lymphoblasts). In addition, myeloid leukemia can be expressed in a chronic way (CML).

Due to rapid uncontrolled development, acute leukemia shows symptoms like anaemia, petechia, infection and bleeding that appear more promptly than seen with chronic leukemia. This type of leukemia is an adult disease and distinguished by slow-growing cells, therefore even untreated people can live for many years without any symptoms [4].

2.4.2 Etiology and epidemiology

The exact cause of leukemia is still unknown and depends on the type of leukemia. Not only inherited but also environmental factors seem to be involved in the genesis of the disease. Besides, risk factors like smoking, ionizing radiation, some chemicals, Down syndrome and the family historiy of leukemia play a role in the genesis. From 2002 to 2001, the incidence rate of leukemia has increased 0.2% each year, while the mortalitiy rate has fallen about 1% each year. Around a third of all cancers diagnosed in children are leukemias, despite more than 9 in 10 cases are diagnosed in adults. ALL is the most common type of leukemia in children, being diagnosed in about four fifths of all leukemia cases [32]

2.4.3 Diagnosis and treatment

Following the appearance of symptoms, the common way to diagnose leukemia is based on repeated complete blood counts and a bone examination. In early stages of the disease or during remission, the blood test may lead to untruly results. Further diagnosis by means of blood chemistry tests can be used to identify the degree of liver and kidney damage. Also X-ray, MRI, ultrasound or, rarely, CT-scans, may be used to detect other damages.

The aim of treatment is to induce a lastly remission, which means absence of detectable cancer cells in the body, the return of normal bone marrow cells and normal blood counts. The type of treatment depends on the type of leukemia, the progression of the disease and the premedical history as well as the age of the patient [33].

Chronic Leukemia

Firstly, it is important to find out if the cancer has dispersed in the blood and bone marrow. In general, the treatment intends more to palliate the symptoms than to heal up the disease. Thus, before starting a chemotherapy, the patients' condition will be closely monitored and until signs or symptoms appear, no treatment will be started [4].

Therapy of chronic leukemia has a variety of therapeutic options, including steroids, alkylating agents, purine analogs, monoclonal antibodies, and transplant options [34]. The most common treatment for chronic leukemia is the chemotherapy drug fludarabine. It has been shown that the rate and length of remission was increased by combining fludarabine with Rituximab, a monoclonal antibody. By adding cyclophosphamide to the combination of fludarabine and Rituximab, an even higher response rate was observed, but the risk of infection also increased. Chlorambucil in combination with Bendamustine and Lenalidomid are also approved for chronic leukemia [35].

Acute Leukemia

To choose the best kind of treatment, it is essential to differentiate between AML and ALL. The main treatment for ALL in adults involves long-term use of chemotherapy. Different combinations of chemotherapeutics may be employed, typically including vincristine, dexamethasone, or daunorubicin, or a similar anthracycline drug. Depending on the patients' prognosis, some treatments may also include cyclophosphamide, L-asparaginase, etoposide or high doses of methotrexat or cytarabine (AraC) [4].

The treatment of AML usually has two phases. The first phase is called remission induction therapy. Daunorubicin in combination with cytarabine remains the standard induction chemotherapy combination for patients with AML. The goal is to kill the leukemia cells in the blood and bone marrow.

The second phase, called post-remission therapy or remission continuation therapy, begins after the disease is trapped in remission. Its aim is to extend the complete remission as long as possible [36].

Acute Promyelocytic Leukemia

Genetics play an increasingly important role in the risk stratification and management of AML patients. Most of the prognostic cytogenetic abnormalities in AML are either chromosomal rearrangements or large genomic deletions [37]. Acute Promyelocytic leukemia (APL) constitutes a subtype of AML and is characterized by a chromosomal translocation involving the retinoic acid receptor-alpha gene on chromosome 17 (RARA). RARA is important for the regulation of gene expression. Activated by retinoic acid, RARA is able to bind on the nuclear corepressor factor, which results in differentiation of promyelocytic cells. Without retinoic acid, RARA is bound by nuclear corepressor factor which leads to transcriptional repression.

In large part of APL, chromosome 17 is translocated with the promyelocytic gene (PML) on chromosome 15. As a result, an abnormal fusion protein called PML-RARA is generated. This protein causes an intensified binding from the retinoic acid receptor to the nuclear co-repressor factor. Thus, physiologic doses of retinoic acid are not able anymore to activate the gene, which causes transcriptional repression. Due to that, all-trans acid (ATARA) is used for induction therapy with anthracycline-based chemotherapy to induce remission [39, 40].

For patients with relapsed APL by treatment with antracycline-based therapy, the combination of arsenic trioxide with ATRA was approved for treatment, as it effectively induces remission in the majority of these patients [38, 40].

Use of high-dose Arabinofuranosylcytosine (AraC) is a standard consolidation therapy in AML patients aged <60 years [41]. AraC is the most active drug in treatment of APL [42].

Various RR inhibitors caused synergism together with AraC. Accordingly, an important focus of modern drug design lies on developing combination therapies of such agents with classic treatment regimes.

2.4.4 In vitro cell system

The human HL-60 promyelocytic leukemia cell line has been obtained from a 36-year-old Caucasian woman and was used for all experiments being presented in this thesis [26].

2.5 Thiosemicarbazones

2.5.1 Biological application and mode of action

Thiosemicarbazones contain a sulfur atom instead of the oxygen atom being present in semicarbazones. They are classified into four types, based on the participating aldehyde or ketone in the condensation process: Aldehydes, substituted aldehydes, ketones or substituted ketons with thiosemicarbazides [43].

Depending on their parent type of aldehyde or ketone, thiosemicarbazones and their derivates are unidentate, bidentate or multidentate chelators of metal ions, which are able to form biologically active complexes. This results in antiviral, antibacterial, antifungal, and antitumoral activity. Thiosemicarbazones can disturb the function of RR by binding to the nitrogen bases of DNA and/or by hindering or blocking base replication. Thus, thiosemicarbazones are able to inhibit the synthesis of DNA. In some cases the activity is associated with Fe [43] . After absorption in the small intestine, Fe is transported from the duodenal enterocytes into the blood, where it binds to an iron-binding protein named transferrin. Transferrin is able to bind two Fe-atoms with high affinity and is essential for functions such as erythropoiesis and storage in ferritin. The transferrin receptor 1 (TfR1), which is located on the plasma membran, is able to bind two transferrin molecules to facilitate iron uptake by cells. The transferrin-TfR1 complex gets internalized into the endosome, where the ferric iron is released from transferrin. Numerous studies have demonstrated that cancer cells have higher TfR1 levels compared to normal cells, and that higher TfR1 expression is correlated with more advanced cancers [44].
This sensitivity probably exists because cancer cells have greater Fe requirements than their normal counterparts and express higher levels of the Fe-containing enzyme, RR [45]. As already mentioned, RR is required for de novo synthesis of deoxyribonucleotides and DNA replication and repair which is the critical rate-limiting step in DNA synthesis. In addition, Fe-depletion by chelators has been proven to influence the expression of a number of cyclins and CDKs. It was demonstrated that in prostate and pancreatic cancer cells the expression of different cyclins were downregulated by iron chelators. Thus, thiosemicarbazones are of remarkable interest in the field of chemistry, pharmacology, and tumor biology [46].

2.5.2 Synthesis and structures

Thiosemicarbazones were prepared by condensing acetylpyrazine or a ring-substituted acetylpyrazine with thiosemicarbazide. Using the same procedure, *N*,*N*-dimethylthiosemicarbazones were synthesized from acetylpyrazines and *N*,*N*-dimethylthiosemicarbazide. Thiosemicarbazones can also be synthesized by letting a methyl ester of imidopiolinic acid and a thiosemicarbazide react [46].

Their structure contains a so-called Schiff base (a sulfur atom and an azomethinic nitrogen atom), which is able to function as chelating ligand by forming a five membered ring. This ring allows to chelate transition metal ions, such as Cu (II), Fe (II)/ (III) and Zn (II). Due to the hydrazinic nitrogen, which depronates after complexation, a bond delocalization and lengthening of the C-S bond takes place. This produces the apparently masked thiolate group [47].

2.5.3 VG-series

Qualitative and quantitative structure-activity relationship (QSAR) studies were used to find connectios between chemical structures and biological activities of novel compounds. On the basis of the best QSAR model, 40 novel compounds were synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of technology, Mesra, India. All of the compounds have potential ribonucleotide reductase inhibiting properties. Cell counts and IC_{50} values were determined after 24, 48 and 72, hours and the twelve most promising substances were chosen for further testing.

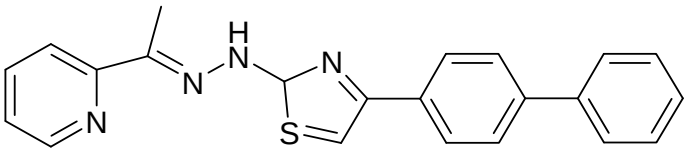
3. Materials and Methods

3.1 Materials

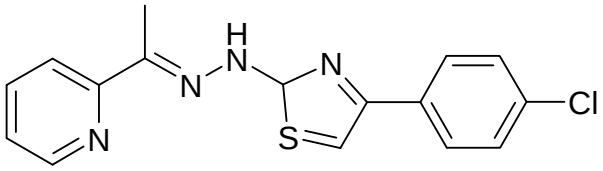
3.1.1 Chemicals and supplies

VG-12, VG-24, and VG-53 were synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, India. Structural formulas are shown in Fig**.** 7.

A



B



C

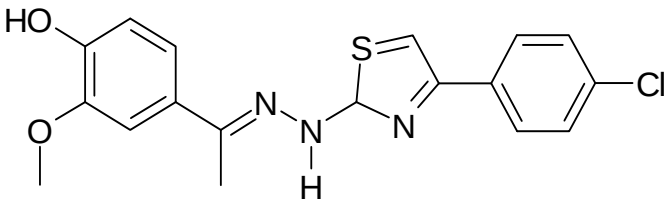


Fig. 7: Chemical structures of VG-12 (A), VG-24 (B), and VG-53 (C)

Cytarabine (AraC) 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 and materials were purchased as described below:

Acetonitrcile	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
Guanosine 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 Pharmacia, 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. Devices

The following devices were used for all experiments:

BD 6220 Incubator	Heraeus, Vienna, Austria
Biometra WT12	CORE Life Sciences
Bioshake IQ	Q. Instruments GmbH
Microcellcounter CC-110	SYSMEX, Kobe, Japan
Centrifuge 5415R	Eppendorf, Vienna
Hettich Rotanta Centrifuge	Hettich Zentrifugen, Tuttlingen
Hitachi U-2000 Spectrophotometer	Hitachi, Austria
Laminar Air Flow	DI W. Ehret GmbH., Emmendingen
MiniSpinPlus	Eppendorf, Vienna
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

3.1.3 Cell culture media and supplements

All media and supplements were obtained from Gibco Life Technologies, Ltd. (Paisley, Scotland, Great Britain):

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

3.1.4 Cell line

The human HL-60 promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 medium with L-Glutamine supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-Glutamine and 1% Penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria). Cell counts were determined using a microcellcounter CC-110 (SYSMEX, Kobe, Japan). Cells growing in the logarithmic phase of growth were used for all experiments described below.

3.2 Methods

3.2.1 Growth inhibition assay

According to a method described by Graser et al [48], HL-60 cells were seeded at a concentration of $0.1x10^6$ per ml in $25cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of drugs at 37°C under cell culture conditions. Stock solutions were diluted in DMSO. Cell counts and IC_{50} values (IC_{50}= 50% growth inhibition of tumor cells) were determined after 24, 48, and 72 hours using the microcellcounter CC-110. Viability of cells was determined by trypan blue staining. Results were calculated as number of viable cells. All experiments were performed in triplicate [48].

3.2.1.1 Simultaneous growth inhibition assay

To investigate the combination effect of VG-12 with AraC, HL-60 cells (0.10×10^6 per ml) were simultaneously incubated with various concentrations of VG-12 and AraC at 37°C under cell culture conditions. After 24, 48, and 72h, cells were counted using the microcellcounter CC-110 [48].

3.2.1.2 Sequential growth inhibition assay

HL-60 cells ($0.1x10^6$ per ml) were first incubated with different concentrations of VG-12 for 24 hours. Then VG-12 was washed out and cells were further exposed to various concentrations of AraC for another 24, 48, and 72 hours. After each period, cells were counted using the microcellcounter CC-110 [48].

3.2.2 Cell cycle distribution analysis

After a method described by Graser et al [49], cells ($0.4x10^6$ per ml) were seeded in $25cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of drugs at 37°C under cell culture conditions. After 24 hours, cells were harvested and suspended in 5ml cold PBS, centrifuged, resuspended and fixed in 1ml cold ethanol (70%) for 30 min at 4°C. After two washing steps in cold PBS, RNAse A was added to a final concentration of 50µg/l and incubated for 60 minutes at 37°C. Subsequently, propium iodide was added to the same concentration and the samples were incubated at 4°C over night. Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated using the ModFit LT software package (Verity Software House, Topsham, ME, USA) [49].

3.2.3 Incorporation of ^{14}C-labeled cytidine into DNA (DNA synthesis assay)

To analyse the effect of drug incubation on the activity of DNA synthesis, an assay was performed as described from Szekeres et al [50]. Logarithmically growing HL-60 cells ($0.3x10^6$ per ml) were incubated with 2, 4, and 6 µM of VG-12 for 24 hours. After the incubation period, cells were counted and pulsed with ^{14}C-cytidine (0.3125µCi, 5nM) for 30 min at 37°C. Then cells were collected by centrifugation and washed with PBS. Total DNA was extracted from $5x10^6$ cells by phenol-chloroform-isoamyl alcohol extraction and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (PerkinElmer, Boston, MA) [48].

3.2.4 Determination of deoxyribonucleoside triphosphates (dNTPs)

Logarithmically growing HL-60 cells were incubated with increasing concentrations of drugs for 24 hours. Afterwards, $5x10^7$ cells were separated for the extraction of dNTPs according to a method initially described by Garrett et al [51]. Cells were centrifuged at 15000 rpm for 5 min and then resuspended in 100µl phosphate-buffered saline. In this suspension, cells were lysed by addition of 10µl trichloroacetic acid and the mixture was vortexed for 1 minute. The lysate was rested on ice for 30 min and then the protein was separated by centrifugation at 15000 rpm for 5 min in an Eppendorf microcentrifuge. The supernatant was removed and neutralized by adding 200µl Freon/TNO (7.8 Freon plus 2.2 TNO) containing 0.5 M tri-n-octylamine. Aliquots of 100µl were periodated by adding 30µl of 4M methylamine solution and 10µl sodium periodate solution (concentration: 100g/l). After incubation at 37°C for 30 min, the reaction was stopped by adding 5µl of 1M rhamnose solution. The extracted dNTPs were measured using a Merck „La Chrom" HPLC system equipped with L-7200 autosampler, L-7100 pump, L-7400 UV detector, and D-7000 interface. Samples were eluted with a 3.2M ammonium phosphate buffer, pH 3.6 (pH adjusted by addition of 0.32M H_3PO_4), containing 20mM acetonitrile using a 4.6x250mm Partisil 10 SAX analytical column (Whatman Ltd., Kent, UK). Separation was performed at constant ambient temperature with a flow rate of 2ml/min. The concentration of dNTPs was calculated as percent of total area under the curve for each sample [48].

3.2.5 Western Blotting

In agreement with a method described by Graser et al [48], HL-60 cells were seeded in $25cm^2$ tissue culture flasks at a concentration of 2×10^6 per ml medium and incubated with 6µM VG-12 for 0.5, 2, 4, 8, and 24 hours.

After the incubation period cells were harvested, washed twice with ice-cold PBS (pH 7.2) and lysed in a buffer containing 150mM NaCl, 50mM Tris-buffered saline (Tris pH 8.0), 1% Triton X-100, 1mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (PIC; from a 100x stock). The lysate was centrifuged at 12000 rpm for 20 min at 4°C, and the supernatant was stored at -20°C until further analysis Equal amounts of lysate (protein samples) were loaded onto polyacrylamide gels. Proteins were electrophoresed (PAGE) at 120 volt (50mA) for approximately 1 hour and then electroblotted onto PVDF membranes (Hybond P, Amersham, Buckinghamshire, UK) at 100 V and 4ºC for 60 min.

Equal sample loading was controlled by staining membranes with Ponceau S. After washing with PBS/Tween-20 (PBS/T) pH 7.2 or Tris/Tween-20 (TBS/T) pH 7.6, membranes were blocked for 60 min in blocking solution (5% non-fat dry milk in PBS containing 0.5% Tween-20).

Then membranes were incubated with the first antibody (in blocking solution, dilution 1:500 to 1:1000) at 4°C, overnight. Subsequently, the membranes were washed with PBS or TBS and further incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG dilution 1:2000 to 1:5000 in PBS/T or TBS/T) at room temperature for 60 min.

Membranes were washed with PBS/Tween-20 (PBS/T) pH 7.2. Chemoluminescence was developed by the ECL detection kit (Amersham, Buckinghamshire, UK) and then membranes were exposed to Amersham Hyperfilms [48].

Antibodies

The following antibodies were used:

Anti-β-Actin	#A1978, monoclonal clone AC-15m mouse ascites fluid (Sigma Aldrich)
Chk1	#2345, polyclonal (Cell Signaling)
Chk2	#2662, polyclonal (Cell Signaling)
Phospho-ATR	#2853, polyclonal (Cell Signaling)
P53R2	#sc-10840, polyclonal (Santa Cruz)
R1	#sc-11733, polyclonal (Santa Cruz)
R2	#sc-10848, polyclonal (Santa Cruz)
Donkey anti-goat IgG-HRP	#sc-2020, polyclonal (Santa Cruz)
Anti Rabbit Immuno HRP	#PO217, polyclonal (Dako)

3.2.6 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. The calculations of dose response curves and combination effects were performed using the "Calcusyn" software designed by Chou and Talalay (Biosoft, Ferguson, MO) [48].

4. Results

4.1. Effect of VG-12, VG-24, and VG-53 on the growth of HL-60 cells

HL-60 cells were seeded in 25cm^2 tissue culture flasks at a concentration of 0.1x10^6 per ml and incubated with increasing concentrations of VG-12, VG-24, and VG-53 at 37°C. After 72 hours, the cell number of viable leukemia cells was determined. After 48h, VG-12, VG-24 and VG-53 inhibited the growth of HL-60 cells with IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) of 1.5, 2.6, and 4.9 µM, respectively. After 72h, VG-12, VG-24, and VG-53 yielded IC_{50} values of 1.4, 1.8 and 3.9 µM after 72h, respectively (see table 2). The cell number of viable leukemia cells were determined using a microcellcounter CC-110. All further investigations were performed with the most promising compound, VG-12.

Compound	MW	IC_{50} (48h)	IC_{50} (72h)
VG-12	371.00	1.5 µM	1.4 µM
VG-24	343.80	2.6 µM	1.8 µM
VG-53	373.86	4.9 µM	3.9 µM

Tab. 2: Effect of VG-12, VG-24, and VG-53 on the growth of HL-60 cells

4.2. Effect of VG-12 on the growth of HL-60 cells

HL-60 cells were seeded at a concentration of 0.1x10^6 per ml and incubated with increasing concentrations of VG-12. After 24, 48, and 72 hours the cell number of viable leukemia cells was determined. VG-12 inhibited the growth of HL-60 cells with IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) of 1.5 and 1.4 µM after 48 and 72 hours, respectively (Fig. 8).

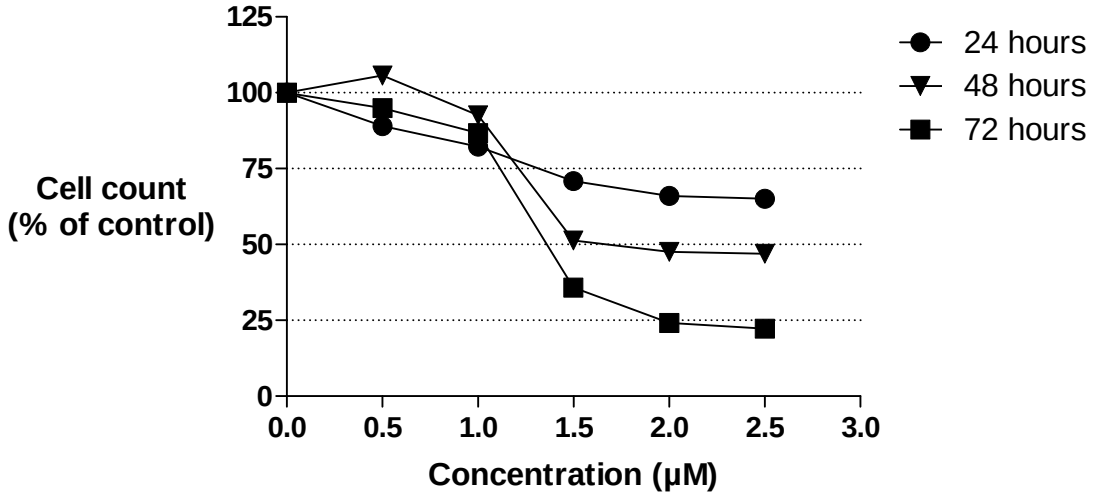


Fig. 8: Growth inhibition of HL-60 cells after incubation with VG-12.

4.2.1 Synergistic combination effects of VG-12 and AraC on the growth of HL-60 cells

To investigate the effect of VG-12 in combination with AraC, HL-60 cells were seeded at a concentration of $0.1x10^6$ per ml and simultaneously or sequentially (Tab. 4 and Tab. 5) incubated with increasing concentrations of drugs (VG-12 first for 24 hours and then AraC for 48 hours as described in the methods section). None of the combinations caused synergistic effects on the growth of HL-60 cells when applied simultaneously, whereas when applied sequentially, 4 out of 9 drug combinations showed synergistic combination effects after treatment with VG-12 for 24h and AraC for 48h. Likewise, 4 out of 9 drug combinations yielded highly synergistic effects after treatment with VG-12 for 24h and with AraC for 72h (Tab. 4 and Tab. 5).

Tab. 3: Synergistic combination effects of VG-12 and AraC in HL-60 cells employing a simultaneous growth inhibition assay. Cells were simultaneously incubated with VG-12 and AraC for 24h, 48h, and 72h, and then cell numbers were determined. Data are means of three independent determinations. Standard deviations (SD) were within 5%.

		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*
VG12 (μM)	1	95.8		102.0		95.8	
	2	81.7		74.1		71.8	
	3	75.5		46.5		33.0	
AraC (nM)	10	61.1		56.3		58.7	
	15	54.9		51.3		48.5	
	20	51.6		44.3		37.1	
VG12 + AraC	1 + 10	60.1	**1.189**	52.2	**1.150**	53.0	**1.225**
VG12 + AraC	1 + 15	52.3	**1.010**	45.0	**1.116**	41.9	**1.215**
VG12 + AraC	1 + 20	52.0	**1.251**	38.0	**1.006**	31.5	**1.112**
VG12 + AraC	2 + 10	56.2	**1.160**	39.9	**1.085**	36.9	**1.164**
VG12 + AraC	2 + 15	52.3	**1.217**	35.5	**1.121**	28.6	**1.146**
VG12 + AraC	2 + 20	50.3	**1.328**	30.6	**1.088**	22.5	**1.107**
VG12 + AraC	3 + 10	54.6	**1.284**	30.5	**1.228**	17.3	**0.971**
VG12 + AraC	3 + 15	52.3	**1.425**	29.1	**1.291**	16.4	**1.048**
VG12 + AraC	3 + 20	50.0	**1.502**	26.5	**1.303**	14.0	**1.053**

* Combination indices according to the equation of Chou and Talalay (Chou and Talalay, 1981)

Tab. 4: Synergistic combination effects of VG-12 and AraC in HL-60 cells employing a sequential growth inhibition assay. Cells were sequentially incubated with VG12 (A) for 24h and AraC (B) for 48h, and then the cell numbers were determined. Data are means of three determinations. Standard deviations were within 5%.

Compound	Concentration (µM/nM)	Cell number (% of control)	Predicted value*	Combination Index**
VG12 (A) (µM)	1.0	87.6		
	2.0	46.4		
	3.0	30.6		
AraC (B) (nM)	10	80.7		
	15	78.8		
	20	77.1		
VG12 + AraC	1.0 10	70.9	70.6	**0.857*** **
VG12 + AraC	1.0 15	71.3	69.0	**0.964**
VG12 + AraC	1.0 20	61.5	67.5	**0.671*** **
VG12 + AraC	2.0 10	41.6	37.4	**0.852*** **
VG12 + AraC	2.0 15	37.2	36.5	**0.793*** **
VG12 + AraC	2.0 20	34.3	35.8	**0.755**
VG12 + AraC	3.0 10	27.9	24.7	**1.005**
VG12 + AraC	3.0 15	27.2	24.1	**0.992**
VG12 + AraC	3.0 20	26.2	23.6	**0.972**

* Predicted Value: (%A x %B) / 100

** Combination indices according to the equation of Chou and Talalay (Chou and Talalay, 1981)

*** Synergistic combination effect

Tab. 5: Synergistic combination effects of VG-12 and AraC in HL-60 cells employing a sequential growth inhibition assay. Cells were sequentially incubated with VG12 for 24h and AraC for 72h, and then the cell number was measured. Data are means of three independent determinations. Standard deviations were within 5%.

Compound	Concentration (µM/nM)	Cell number (% of control)	Predicted value*	Combination Index**
VG12 (µM)	1.0	97.9		
	2.0	39.6		
	3.0	18.3		
AraC (nM)	10	86.1		
	15	82.4		
	20	79.6		
VG12 + AraC	1.0 10	78.2	84.3	**1.068**
VG12 + AraC	1.0 15	75.2	80.7	**1.120**
VG12 + AraC	1.0 20	60.9	77.9	**0.785*** **
VG12 + AraC	2.0 10	31.7	34.1	**0.852*** **
VG12 + AraC	2.0 15	27.4	32.6	**0.820*** **
VG12 + AraC	2.0 20	23.1	31.5	**0.784*** **
VG12 + AraC	3.0 10	15.4	15.7	**1.038**
VG12 + AraC	3.0 15	15.4	15.1	**1.042**
VG12 + AraC	3.0 20	12.1	14.6	**0.994**

* Predicted Value: (%A x %B) / 100

** Combination indices according to the equation of Chou and Talalay (Chou and Talalay, 1981)

*** Synergistic combination effect

4.3 Inhibition of incorporation of ^{14}C-labeled cytidine into DNA of HL-60 cells after treatment with VG-12

HL-60 cells were seeded at a concentration of $0.4x10^6$ cells per ml. Exposure to 2, 4, and 6 µM of VG-12 for 24 hours significantly decreased ^{14}C-Cytidine incorporation into nascent DNA of HL-60 cells to 73, 48, and 30% of untreated controls, respectively (Fig.9). This indicates a reduced cytidine metabolism, being equivalent to an inhibition of RR *in situ* activity.

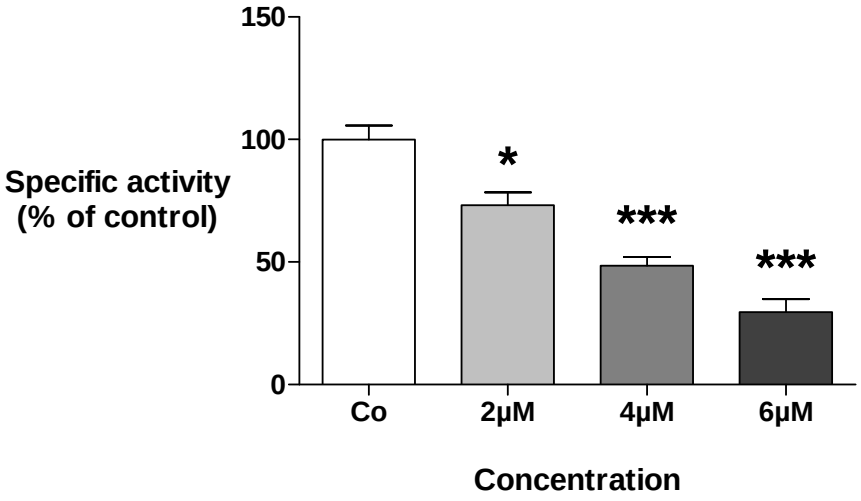


Fig. 9: *In situ* measurement of ribonucleotide reductase activity in HL-60 cells after treatment with VG-12 for 24 hours

4.4 Alterations of dNTP pool sizes in HL-60 cells after treatment with VG-12 for 24 hours

Constitutive RR activity maintains balanced dNTP pools, whereas RR inhibition tilts this balance. In line with the inhibition of RR *in situ* activity, VG-12 treatment caused also an imbalance of dNTPs in HL-60 cells after 24 hours, which was determined by HPLC analysis. Incubation of cells with 2µM VG-12 resulted in a significant depletion of intracellular dCTP pools to 87% of controls. After treatment with 4µM VG-12, the dCTP and dATP pools decreased to 70% and 80% of controls, respectively. Treatment with 6 µM VG-12 caused a significant depletion of dCTP, dTTP, and dATP concentrations to 59%, 77%, and 57% of untreated controls, respectively (Fig. 10).

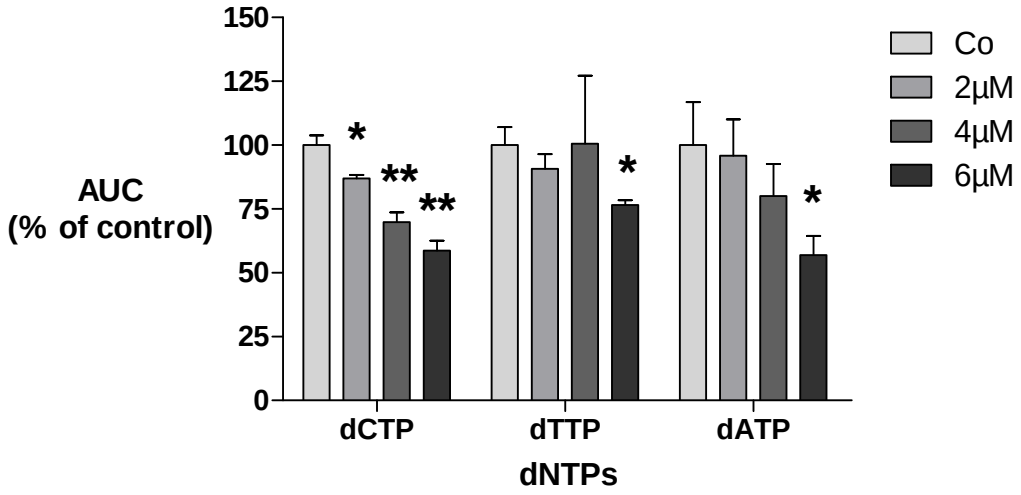


Fig. 10: Concentration of dNTP pools in HL-60 cells after treatment with VG-12 for 24 hours

4.5 Expression of RR subunits R1, R2, and p53R2 after treatment with VG-12

To monitor the effect of RR inhibitors on the expression of RR subunits, HL-60 cells were incubated with 6µM VG-12 for 0.5, 2, 4, 8, and 24 hours and subjected to western blot analysis. The protein level of the constitutively expressed R1 subunit was slightly elevated after 8h, whereas R2 was significantly down regulated after 24h. P53R2 protein levels increased subtly after 8h incubation with VG-12 (Fig. 11)

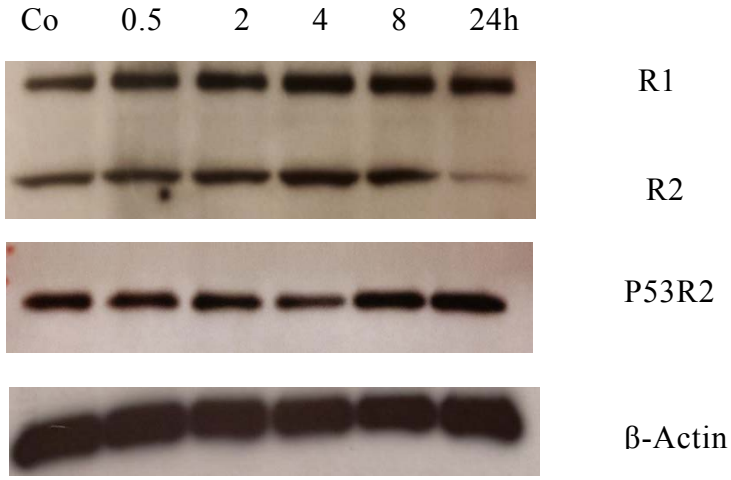


Fig. 11: Expression of RR subunits in HL-60 cells after treatment with VG-12

4.6 Cell cycle distribution analysis after treatment with VG-12

HL-60 cells were simultaneously incubated with 6µM VG-12 and/or 15nM AraC for 24 hours. Treatment of HL-60 cells with 6 µM VG-12 only marginally affected S-phase cells, increasing this cell population from 48 to 51%, whereas G0-G1 phase cells decreased from 35 to 31%. (Fig. 12)

Incubation with 20nM AraC caused an accumulation of 60% HL-60 cells in S-phase and a concomitant decrease of G0-G1 cells to 20%. (Fig. 13)

Simultaneous incubation of HL-60 cells with 4µM VG-12 and 15nM AraC led to an even more pronounced S-phase arrest, increasing this cell population from 46 to 80% while decreasing cells in the G0-G1 phase from 46 to 15% (Fig. 13).

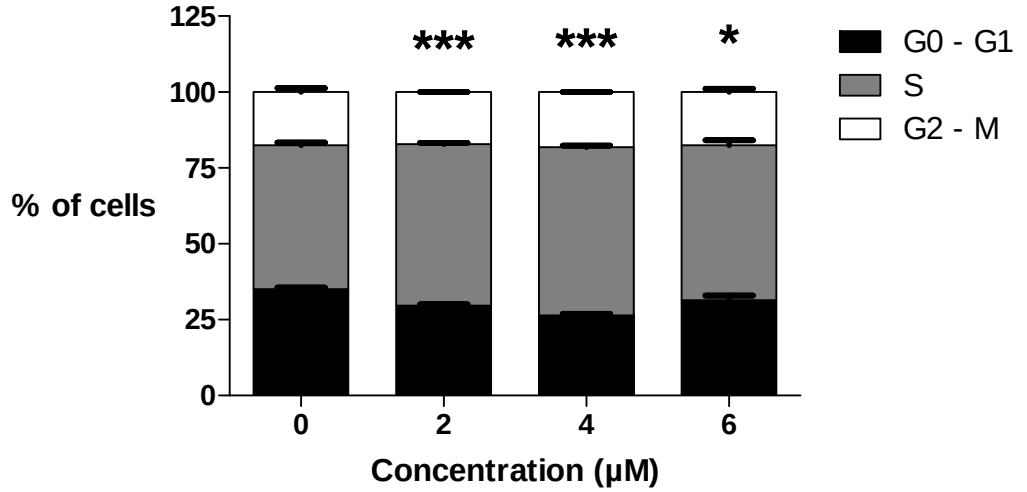


Fig. 12: HL-60 cell cycle distribution after incubation with VG-12

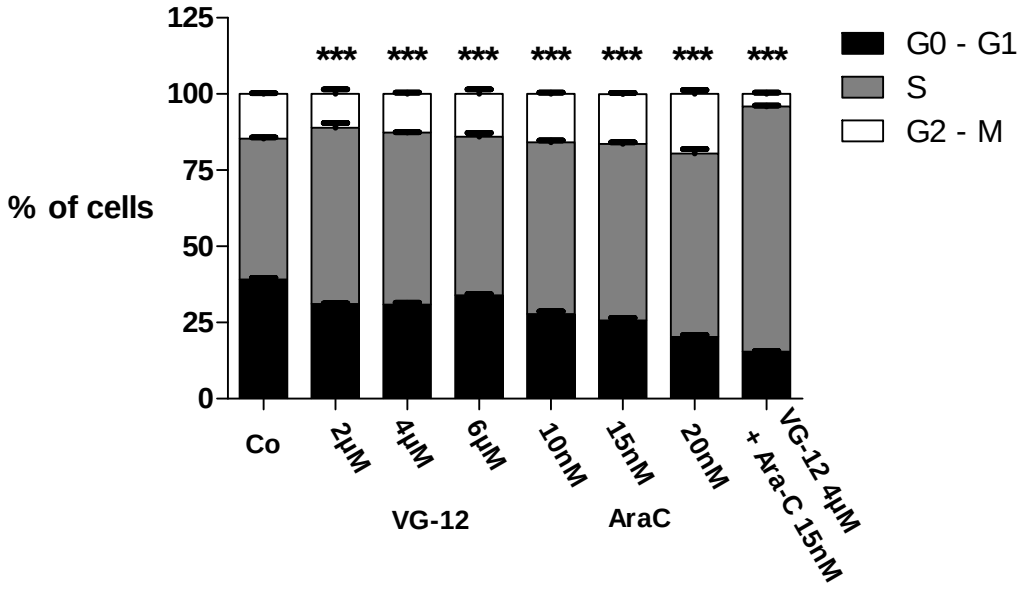


Fig. 13: HL-60 cell cycle distribution after simultaneous incubation with VG-12 and AraC

4.7. Expression of checkpoint and cell cycle regulating proteins after treatment with VG-12

To investigate whether S-phase inhibition caused activation of cell cycle checkpoint kinases, HL-60 cells were simultaneously treated with 6μM VG-12 for 0.5, 2, 4, 8, and 24 hours and subjected to western blot analysis. Chk1, ATR, and Chk2 levels were all slightly elevated after 4, 8 and 24 hours (Fig.14).

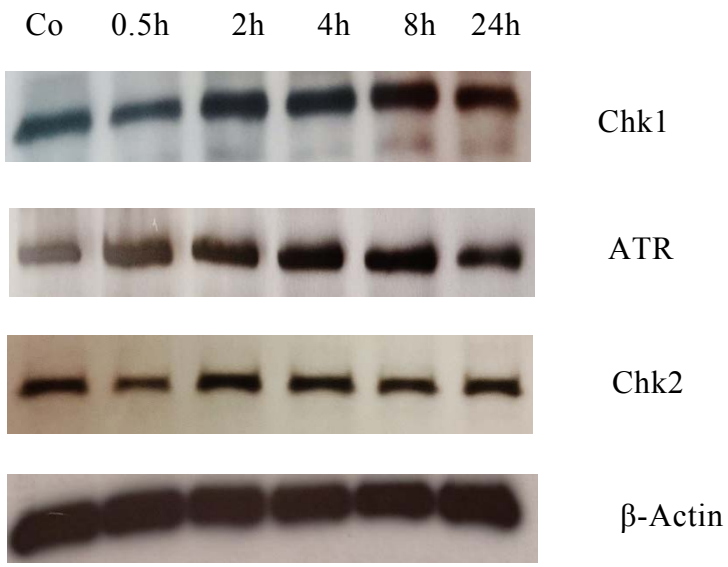


Fig. 14: Expression of checkpoint and cell cycle regulators upon treatment with VG-12

5. Discussion

A panel of 40 thiosemicarbazone derivatives was designed to inhibit the enzyme ribonucleotide reductase (RR) activity using 3D molecular space modeling techniques. RR represents the rate-limiting factor of DNA synthesis. Growth inhibition assays in human HL-60 promyelocytic leukemia cells with all newly designed agents revealed that VG-12 was the most active compound yielding the lowest IC_{50} values. Furthermore, VG-12 was combined with arabinofuranosylcytosine (AraC), a well-established first-line antitumor agent. In general, sequential combination of numerous anticancer drugs with AraC potentiates the antiproliferative effect of AraC synergistically [42]. Indeed, employing a sequential growth inhibition assay with VG-12 and AraC, 4 out of 9 concentrations caused synergistic effects after an incubation time of 48 and 72 hours. In contrast, no synergistic effect could be observed by simultaneous inhibition assays.

Besides, VG-12 was proved to be a powerful inhibitor of RR *in situ* activity after incubation time of 24 hours. Treatment with 6 µM of VG-12 caused a decrease to 30% of cell count compared to untreated controls. In addition, this newly designed agent led to alterations of deoxyribonucleoside triphosphate (dNTP) pool balance. dCTP, dTTP, and dATP pools were significantly depleted.As a consequence of such an imbalance, *de novo*DNA synthesis is disturbed and cell proliferation blocked.

Treatment with 6µM VG-12 marginally affected G0-G1 phase cells, whereas S-phase cells slightly increased, which is correlative with important RR properties: The enzyme forms the rate limiting factor for S-phase transit to G2-M phase. Inhibition of RR leads to arrest of cells in S-phase[52]._This corresponds to our finding that the S-phase specific R2 subunit was significantly down regulated by treatment with 6 µM VG-12.

Combination treatment with AraC led to fortified effects in cell cycle progression. Simultaneous incubation of 4µM VG-12 and 15 nM AraC led to an S-phase arrest, increasing this cell population from 46% to 80% as compared to untreated controls while decreasing cells in G0-G1 phase from 46% to 15%.

Before mitosis, cells have to pass the three major cell cycle checkpoint kinases G1-S, intra-S, and G2-M. Cell cycle checkpoint kinases are controlled by their regulators, ATR and ATM kinases. The latter are activated by their downstream effector kinases Chk1 and Chk2, respectively, which are up-regulated by incomplete DNA replication, DNA damage or imbalance of dNTP pools. This clearly defined system allows control at cell progression and repair DNA before damage gets passed on to daughter cells [10].

After treatment with 6 µM VG-12 for 0.5 hours, expression of R1 was slightly elevated, and the p53R2 subunit protein of RR was even clearly increased. These findings suggest the assumption that cells try to rebalance the dNTP production by a compensatory up-regulation of p53R2 [53]. In addition, it was demonstrated that the up-regulation of R1 correlates with DNA damage, which led to up-regulation and activation of Chk1 [54]. Treatment with 6µM VG-12 resulted in a slightly activation of ATR after 2 hours, which led to an up-regulation of Chk1. This corresponds to the activation of Chk2 after treatment with VG-12 for 2 hours.

Taken together, we could demonstrate that VG-12 exerts remarkable anticancer effects in HL-60 cells, both as single drug and in combination with AraC. VG-12 was revealed to be a potent inhibitor of cell cycle progression in S-phase and an effective RR inhibitor, which perturbed dNTP pool balance.

Regarding the synergistic effects with AraC, VG-12 is considered as a potent candidate for future cancer treatment options. Due to these promising results, further investigations and in vivo studies with these novel anticancer drugs are warranted.

6. Abstract

Cancer is a general term for related diseases, which are characterised by uncontrolled expression and proliferation of malignant tumor cells. Due to their increased need of DNA, the expression level of enzyme ribonucleotide reductase (RR), which is responsible for the building bricks of DNA synthesis and DNA repair, is up-regulated. Because RR is able to convert ribonucleoside diphosphate into desoxyribonucleoside diphosphate, it is the rate limiting enzyme for *de novo* dNTP synthesis. As a result, RR is considered to be a sensitive target for chemotherapeutic agents. Due to the latter fact and the urgent need for new cancer treatments, RR is essential for further discoveries and developments of novel compounds

In this diploma thesis, different thiosemicarbazone derivatives have been investigated. Regarding their ability to inhibit the activity of RR, they were supposed to possess anticancer activity. Biochemical effects were analysed in human HL-60 promyelocytic leukemia cells. Considering the results of different growth inhibition assays, the molecule with the lowest IC_{50}, VG-12, was chosen to be analysed more detailed.

This novel agent proved to be an effective inhibitor of RR by significantly lowering the incorporation of ^{14}C-labeled cytidine into DNA of tumor cells. Furthermore, treatment with VG-12 caused an alteration of dNTP pool balance. In addition, it was shown that VG-12 influences the regulation of cell cycle progression by arresting cells in S-phase, which leads to activation of check point kinases Chk1, Chk2, and ATR kinase.

This correlates with the decrease of the RR subunit called R2, which is responsible for the cell transit from S-phase to G2-M phase. This down-regulation was demonstrated by Western Blot analysis. On the other hand, as a result of the inhibition of RR, an increase of expression levels of R1 and P532 subunits could be shown. This caused an activation of the specific checkpoint kinases Chk1, Chk2, and ATR.

Finally, VG-12 was combined with arabinofuranosylcytosine (AraC), a well-established antitumor agent. Employing sequential growth inhibition assays, synergistic antineoplastic effects were determined. In addition, combination treatment influenced the cell cycle progression and caused an increased arrest of cells in the S-phase.

Due to these promising results, we recommend VG-12 both as single drug and as enhancer of other antileukemic agents such as AraC for further preclinical investigations and *in vivo* studies.

7. Zusammenfassung

Krebs ist ein Sammelbegriff für verschiedene Krankheiten, deren gemeinsames Merkmal die unkontrollierte Expression und Proliferation von Zellen ist. Aufgrund des damit einhergehenden erhöhten Bedarfs an DNA ist das Enzym Ribonukleotidreduktase (RR), das für die Bereitstellung der Bausteine der DNA Synthese und Reparatur verantwortlich ist, in Tumorzellen stark überexprimiert. Aufgrund ihrer Fähigkeit, Ribonukleosiddiphosphat in Desoxyribonukleosiddiphosphat umzuwandeln, fungiert die RR als limitierendes Enzym in der *de novo* DNA Synthese. Daher stellt die RR ein geeignetes Angriffsziel für Zytostatika dar und ist aufgrund des steigenden Bedarfs an neuen Krebstherapien von großer Bedeutung für weitere Entwicklungen.

Im Zuge dieser Diplomarbeit wurden substituierte Thiosemicarbazone untersucht, von denen man sich aufgrund ihrer Fähigkeit, die RR Aktivität in unterschiedlicher Weise zu inhibieren, eine antikanzerogene Wirkung erhoffte. Ihre biochemischen Wirkungen wurden in humanen HL-60 Promyelozytenleukämiezellen erforscht. Als Leitsubstanz wurde VG-12 ausgewählt, das Molekül mit dem niedrigsten IC_{50}-Wert.

Der inhibierende Effekt von VG-12 auf die RR konnte durch die signifikante Abnahme des Einbaus von ^{14}C-markiertem Cytidin in die DNA von Tumorzellen gezeigt werden. Weiters konnte eine deutliche Veränderung der ansonsten ausgewogenen Verteilung der dNTP Konzentrationen nachgewiesen werden.

Weitere Untersuchungen zeigten, dass VG-12 in die Regulation des Zellzyklus eingreift und so eine Anhäufung der Zellen in der S-Phase bewirkt.

Dies korreliert mit der deutlichen Abnahme des Expressionslevels der RR-Untereinheit R2, was durch Western Blotting demonstriert werden konnte. Als Folge der RR Hemmung konnte eine verstärkte Expression der RR-Untereinheiten R1 und P53R2 gezeigt werden. Dies wiederum führt zu einer Aktivierung der beiden spezifischen Checkpoint Effektorkinasen Chk1 und Chk2 bzw. der ATR-Kinase.

Schließlich wurde gezeigt, dass VG-12 in verschiedenen Konzentrationen kombiniert mit Arabinofuranosylcytosin (AraC), einem bereits etablierten zytostatischen Chemotherapeutikum, im Zuge eines sequentiellen Wachstumshemmungsassays, synergistische antineoplastische Effekte erzielt. Auch die Regulation des Zellzyklus wird durch diese Kombinationsbehandlungen beeinflusst und bewirkt eine entsprechend verstärkte Arretierung der Zellen in der S-Phase des Zellzyklus.

Aufgrund dieser vielversprechenden Ergebnisse sollten weitere präklinische Untersuchungen und *in vivo* Studien sowohl mit VG-12 alleine, als auch in Kombination mit AraC durchgeführt werden.

8. References

[1] “http://www.who.int (18.09.2015).” .

[2] P. Saiko, M.-T. Steinmann, H. Schuster, G. Graser, S. Bressler, B. Giessrigl, A. Lackner, M. Grusch, G. Krupitza, Z. Bago-Horvath, W. Jaeger, M. Fritzer-Szekeres, and T. Szekeres, “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*, vol. 22, no. 1, pp. 213–222, Jan. 2015.

[3] A. Urruticoechea, R. Alemany, J. Balart, A. Villanueva, F. Vinals, and G. Capella, “Recent advances in cancer therapy: an overview,” *Curr. Pharm. Des.*, vol. 16, no. 1, pp. 3–10, 2010.

[4] “http://www.cancer.gov (10.10.2015).” .

[5] D. Hanahan and R. A. Weinberg, “Hallmarks of Cancer: The Next Generation,” *Cell*, vol. 144, no. 5, pp. 646–674, Mar. 2011.

[6] Hupé, “Hallmarks of cancer,” “https://en.wikipedia.org, 25.9.2015.” .

[7] G. M. Cooper, “The cell: a molecular approach,” in *The cell: a molecular approach*, 2nd edition., United States: Washington, D.C. : Sunderland, Mass. : ASM Press ; Sinauer Associates, c2000., p. 689.

[8] “http://zebrafish.umdnj.edu/PEnrollment/Resources/Biology/Eukaryotic (25.09.2015).” .

[9] M.-T. Park and S.-J. Lee, “Cell cycle and cancer,” *J. Biochem. Mol. Biol.*, vol. 36, no. 1, pp. 60–65, 2003.

[10] J. Bartek and J. Lukas, “Chk1 and Chk2 kinases in checkpoint control and cancer,” *Cancer Cell*, vol. 3, no. 5, pp. 421–429, 2003.

[11] P. Bouwman and J. Jonkers, “The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance,” *Nat. Rev. Cancer*, vol. 12, no. 9, pp. 587–598, Aug. 2012.

[12] M.-T. Park and S.-J. Lee, “Cell cycle and cancer,” *J. Biochem. Mol. Biol.*, vol. 36, no. 1, pp. 60–65, 2003.

[13] G. Kroemer, L. Galluzzi, P. Vandenabeele, J. Abrams, E. S. Alnemri, E. H. Baehrecke, M. V. Blagosklonny, W. S. El-Deiry, P. Golstein, D. R. Green, M. Hengartner, R. A. Knight, S. Kumar, S. A. Lipton, W. Malorni, G. Nuñez, M. E. Peter, J. Tschopp, J. Yuan, M. Piacentini, B. Zhivotovsky, and G. Melino, “Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009,” *Cell Death Differ.*, vol. 16, no. 1, pp. 3–11, Jan. 2009.

[14] S. W. Lowe and A. W. Lin, “Apoptosis in cancer,” *Carcinogenesis*, vol. 21, no. 3, pp. 485–495, Mar. 2000.

[15] V. Nikoletopoulou, M. Markaki, K. Palikaras, and N. Tavernarakis, “Crosstalk between apoptosis, necrosis and autophagy,” *Biochim. Biophys. Acta BBA - Mol. Cell Res.*, vol. 1833, no. 12, pp. 3448–3459, Dec. 2013.

[16] G. Kroemer and B. Levine, “Autophagic cell death: the story of a misnomer,” *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 12, pp. 1004–1010, Dec. 2008.

[17] D. Lundin, G. Berggren, D. Logan, and B.-M. Sjöberg, “The Origin and Evolution of Ribonucleotide Reduction,” *Life*, vol. 5, no. 1, pp. 604–636, Feb. 2015.

[18] P. Reichard, “Ribonucleotide reductases: Substrate specificity by allostery,” *Biochem. Biophys. Res. Commun.*, vol. 396, no. 1, pp. 19–23, May 2010.

[19] C. Zhang, G. Liu, and M. Huang, “Ribonucleotide reductase metallocofactor: assembly, maintenance and inhibition,” *Front. Biol.*, vol. 9, no. 2, pp. 104–113, Feb. 2014.

[20] “http://web.mit.edu/biochemistry, 25.09.2015.”

[21] P. Nordlund and P. Reichard, “Ribonucleotide reductases,” *Annu Rev Biochem*, vol. 75, pp. 681–706, 2006.
[22] L. Sun and J. A. Fuchs, “Escherichia coli ribonucleotide reductase expression is cell cycle regulated.,” *Mol. Biol. Cell*, vol. 3, no. 10, pp. 1095–1105, 1992.
[23] S. Gon, J. E. Camara, H. K. Klungsøyr, E. Crooke, K. Skarstad, and J. Beckwith, “A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in Escherichia coli,” *EMBO J.*, vol. 25, no. 5, pp. 1137–1147, 2006.
[24] U. Uhlin and H. Eklund, “Structure of ribonucleotide reductase protein R1,” *Nature*, vol. 370, no. 6490, pp. 533–539, Aug. 1994.
[25] V. R. Jonna, M. Crona, R. Rofougaran, D. Lundin, S. Johansson, K. Brännström, B.-M. Sjöberg, and A. Hofer, “Diversity in Overall Activity Regulation of Ribonucleotide Reductase,” *J. Biol. Chem.*, vol. 290, no. 28, pp. 17339–17348, Jul. 2015.
[26] “https://en.wikipedia.org/wiki (01.10.2015).” .
[27] Nuno M.F.S.A. Cerqueira, Susana Pereira, Pedro A. Fern et al, “Overview of Ribonucleotide Reductase Inhibitors: An Appealing Target in Anti-Tumour Therapy,” in *Overview of Ribonucleotide Reductase Inhibitors: An Appealing Target in Anti-Tumour Therapy*, Portugal: Bentham Science Publishers, 2005, pp. 1283 – 1294.
[28] P. Saiko, M. Pemberger, Z. Horvath, I. Savinc, M. Grusch, N. Handler, T. Erker, W. Jaeger, M. Fritzer-Szekeres, and T. Szekeres, “Novel resveratrol analogs induce apoptosis and cause cell cycle arrest in HT29 human colon cancer cells: inhibition of ribonucleotide reductase activity,” *Oncol. Rep.*, vol. 19, no. 6, pp. 1621–1626, 2008.
[29] Z. Horvath, P. Saiko, C. Illmer, S. Madlener, T. Hoechtl, W. Bauer, T. Erker, W. Jaeger, M. Fritzer-Szekeres, and T. Szekeres, “Synergistic action of resveratrol, an ingredient of wine, with Ara-C and tiazofurin in HL-60 human promyelocytic leukemia cells,” *Exp. Hematol.*, vol. 33, no. 3, pp. 329–335, 2005.
[30] J. Shao, B. Zhou, B. Chu, and Y. Yen, “Ribonucleotide Reductase Inhibitors and Future Drug Design,” *Curr. Cancer Drug Targets*, vol. 6, no. 5, pp. 409–431, Aug. 2006.
[31] S. Nyholm, L. Thelander, and A. Gräslund, “Reduction and loss of the iron center in the reaction of the small subunit of mouse ribonucleotide reductase with hydroxyurea,” *Biochemistry (Mosc.)*, vol. 32, no. 43, pp. 11569–11574, 1993.
[32] “http://www.cancerresearchuk.org (02.10.2015).” .
[33] “http://www.cancer.org (01.10.15).” .
[34] “Chronic Lymphocytic Leukemia Treatment (PDQ®): Health Professional Version.” National Cancer Institute, 04-Sep-2015.
[35] “http://www.ucsfhealth.org.” .
[36] E. J. Feldman, “Novel Therapeutics for Therapy-Related Acute Myeloid Leukemia: 2014,” *Clin. Lymphoma Myeloma Leuk.*, vol. 15, pp. S91–S93, Jun. 2015.
[37] S. Yohe, “Molecular Genetic Markers in Acute Myeloid Leukemia,” *J. Clin. Med.*, vol. 4, no. 3, pp. 460–478, Mar. 2015.
[38] Sandy D Kotiah, “http://emedicine.medscape.com (13.10.2015).” .
[39] M. S. Tallman, “All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol,” *Blood*, vol. 100, no. 13, pp. 4298–4302, Dec. 2002.
[40] Powell BL, Moser B, Stock W, et al, “Arsenic trioxide improves event-free and overall survival for adults with acute promyelocytic leukemia: North American Leukemia Intergroup Study.” .
[41] T. Robak and A. Wierzbowska, “Current and emerging therapies for acute myeloid leukemia,” *Clin. Ther.*, vol. 31, pp. 2349–2370, Jan. 2009.

[42] K. B. B. Pagnano, E. M. Rego, S. Rohr, M. de Lourdes Chauffaille, R. H. Jacomo, R. Bittencourt, A. B. Firmato, E. M. Fagundes, R. A. Moraes Melo, and W. Bernardo, "Guidelines on the diagnosis and treatment for acute promyelocytic leukemia: Associação Brasileira de Hematologia, Hemoterapia e Terapia Celular Guidelines Project: Associação Médica Brasileira - 2013," *Rev. Bras. Hematol. E Hemoter.*, vol. 36, no. 1, pp. 71–89, Jan. 2014.
[43] L. N. Suvarapu, A. R. Somala, J. R. Koduru, S. O. Baek, and V. R. Ammireddy, "A critical review on analytical and biological applications of thio-and phenylthiosemicarbazones," *Asian J. Chem.*, vol. 24, no. 5, pp. 1889–1898, 2012.
[44] G. Y. L. Lui, Z. Kovacevic, V. Richardson, A. M. Merlot, D. S. Kalinowski, and D. R. Richardson, "Targeting cancer by binding iron: Dissecting cellular signaling pathways," *Oncotarget Vol 6 No 22*, 2015.
[45] J. Yuan, "Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: in vitro and in vivo assessment," *Blood*, vol. 104, no. 5, pp. 1450–1458, Sep. 2004.
[46] V. Opletalová, D. S. Kalinowski, M. Vejsová, J. Kuneš, M. Pour, J. Jampílek, V. Buchta, and D. R. Richardson, "Identification and Characterization of Thiosemicarbazones with Antifungal and Antitumor Effects: Cellular Iron Chelation Mediating Cytotoxic Activity," *Chem. Res. Toxicol.*, vol. 21, no. 9, pp. 1878–1889, Sep. 2008.
[47] B. M. Paterson and P. S. Donnelly, "Copper complexes of bis(thiosemicarbazones): from chemotherapeutics to diagnostic and therapeutic radiopharmaceuticals," *Chem. Soc. Rev.*, vol. 40, no. 5, p. 3005, 2011.
[48] G. Graser, "Inhibition of ribonucleotide reductase by small molecules," uniwien, 2011.
[49] H. U. K. Schuster, "Inhibition of ribonucleotide reductase by natural compounds: A key anti-tumor mechanism in cancer cells," uniwien, 2013.
[50] T. Szekeres, K. Gharehbaghi, M. Fritzer, M. Woody, A. Srivastava, B. van´t Riet, H. Jayaram, and L. Elford, "Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase. Cancer Chemother Pharmacol.," vol. 34, pp. 63–66, 1994.
[51] C. Garrett and S. Santi, "A rapid and sensitive High Pressure Liquid Chromatography assay for deoxyribonucleotide triphosphates in cell extracts," *Anal Biochem*, vol. 99, pp. 268–273, 1979.
[52] K. Chimploy, G. D. Díaz, Q. Li, O. Carter, W.-M. Dashwood, C. K. Mathews, D. E. Williams, G. S. Bailey, and R. H. Dashwood, "E2F4 and ribonucleotide reductase mediate S-phase arrest in colon cancer cells treated with chlorophyllin," *Int. J. Cancer*, vol. 125, no. 9, pp. 2086–2094, Nov. 2009.
[53] S. Yanamoto, T. Iwamoto, G. Kawasaki, I. Yoshitomi, N. Baba, and A. Mizuno, "Silencing of the p53R2 gene by RNA interference inhibits growth and enhances 5-fluorouracil sensitivity of oral cancer cells," *Cancer Lett.*, vol. 223, no. 1, pp. 67–76, Jun. 2005.
[54] Y.-W. Zhang, T. L. Jones, S. E. Martin, N. J. Caplen, and Y. Pommier, "Implication of Checkpoint Kinase-dependent Up-regulation of Ribonucleotide Reductase R2 in DNA Damage Response," *J. Biol. Chem.*, vol. 284, no. 27, pp. 18085–18095, Jul. 2009.

9. Acknowledgements

First and foremost, I want to thank Univ.-Prof. Mag. pharm. Dr. Walter Jäger (Department of Clinical Pharmacy and Diagnostics, University of Vienna) for being in charge of my master thesis.

I would also like to express my gratitude to Univ.-Prof. Dr. Thomas Szekeres (Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna) who provided all facilities for my diploma thesis in his laboratory.

I owe special thanks to Priv.-Doz. Mag. pharm. DDr. Philipp Saiko for supervising and assisting me in every issue throughout my whole research work. He enabled me to write my diploma thesis on this fascinating subject and assisted me throughout the entire process. At all times, he had a sympathetic ear for me.

Many thanks to Mag. Geraldine Graser, who offered me a warm-hearted welcome in the laboratory. She has always been accommodating, helpful and obliging in all matters and concerns the entire time in the lab.

I am very grateful to my brother Sebastian and my sisters Leni and Amy, who always were at hand with help and advice for me.

Special thanks to my boyfriend Tim for his understanding, encouragement, and love.

Last but by no means least, I am deeply grateful to my beloved parents Gunda and Peter for their never-ending encouragement, patient and unconditionally support throughout my whole life.

10. Curriculum Vitae

PERSONAL INFORMATION

Full name:	Agnes Schönhuber
Date of birth:	November 21, 1990
Place of birth:	Vienna, Austria
Citizenship:	Austrian
Home address:	Schlüsselgasse 8/18
	A-1040 Vienna, Austria

EDUCATION

2009 – today	Studies of pharmacy, University of Vienna
June 09, 2009	Matura (school leaving examination), Vienna
2001 – 2009	High School, Vienna
1997 – 2001	Primary School, Vienna