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„Inhibition of ribonucleotide reductase by natural compounds: A key anti-tumor mechanism in cancer cells“

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Table of contents

1 Project aims 1

2 Introduction 4

2.1 Cancer 4

2.2 Cell cycle and cancer 6

2.2.1 Cell division cycle 6

2.2.2 Cell cycle regulation 6

2.2.3 Cell cycle deregulation in cancer 8

2.3 Cell death and cancer 8

2.3.1 Apoptosis 8

2.3.2 Necrosis 9

2.4 Ribonucleotide reductase 9

2.4.1 General information 9

2.4.2 Classification of RRs 10

2.4.3 Structure 12

2.4.4 Catalytic cycle, radical transfer & storage 13

2.4.5 Allosteric regulation 14

2.4.6 RR and its occurrence during the cell cycle 15

2.4.7 RR and cancer 16

2.5 Leukemia 17

2.5.1 Introduction 17

2.5.2 Acute myeloid leukemia 18

2.5.3 Acute promyelocytic leukemia 20

2.6 Polyphenols 22

2.6.1 General information 22

2.6.2 Polyphenols and Human Health 22

2.6.3 Biological roles of Polyphenols 24

2.6.4 Polyphenols and signaling cascades 25

2.7 Ellagic acid 27

2.8 Epigallocatechin gallate (EGCG) 28

3 Materials and Methods 31

3.1 Materials 31

3.1.1 Chemicals and supplies 31

3.1.2 Devices 32

3.1.3 Cell culture media and supplements 33

3.1.4 Cell lines 33

3.2 Methods 34

3.2.1 Growth inhibition assay 34

3.2.2 Cell cycle distribution analysis 35

3.2.3 DPPH• radical scavenging activity assay 35

3.2.4 Hoechst dye 33258 and propidium iodide double staining 36

3.2.5 Incorporation of ^{14}C-labeled cytidine into DNA (DNA synthesis assay) 36

3.2.6 Determination of deoxyribonucleoside triphosphates (dNTPs)....37
3.2.7 Statistical calculations....38

4 Results....39

4.1 Ellagic acid (EA)....39

4.1.1 Effect of EA on the growth of HL-60 cells....39
4.1.2 Cell cycle distribution analysis after treatment with EA....43
4.1.3 Antioxidant activity of EA....44
4.1.4 Induction of apoptosis in HL-60 cells after treatment with EA....45
4.1.5 Inhibition of incorporation of ^{14}C-labeled cytidine into DNA of HL-60 cells after treatment with EA....45
4.1.6 Alterations in dNTP pools after treatment with EA for 24h....46

4.2 Epigallocatechin gallate (EGCG)....47

4.2.1 Effect of EGCG on the growth of HL-60 cells....47
4.2.2 Cell cycle distribution analysis after treatment with EGCG....49
4.2.3 Antioxidant activity of EGCG....49
4.2.4 Induction of apoptosis in HL-60 cells after treatment with EGCG....50
4.2.5 Inhibition of incorporation of ^{14}C-labeled cytidine into DNA of HL-60 cells after treatment with EGCG....52
4.2.6 Alterations in dNTP pools after treatment with EGCG for 24h....53

5 Discussion....55

6 Abstract....59

7 Zusammenfassung....61

8 References....63

9 Acknowledgements....66

10 Curriculum Vitae....67

Table of figures

FIGURE 1: THE HALLMARKS OF CANCER, COMPRISING SIX ABILITIES ACQUIRED BY CANCER CELLS (HANAHAN & WEINBERG, 2000) 5

FIGURE 2: THE DIFFERENT STAGES OF CELL CYCLE TOGETHER WITH REGULATORY CYCLIN-CDK COMPLEXES (VERMEULEN ET AL., 2003 B) 7

FIGURE 3: RADICAL GENERATION IN RR CLASS I. SUBUNIT R2 PRODUCES THE TYROSYL RADICAL WHICH THEN GENERATES THE THIYL RADICAL ON SUBUNIT R1 (REICHARD, 2010). 11

FIGURE 4: R1 SUBUNIT OF *E. COLI* WITH EACH MONOMER CONTAINING ONE BINDING SITE FOR THE SUBSTRATE, ONE ALLOSTERIC SPECIFICITY SITE AND ONE ALLOSTERIC ACTIVITY SITE (REICHARD, 2010). 13

FIGURE 5: STRUCTURE OF ELLAGIC ACID (MERTENS-TALCOTT & PERCIVAL, 2005) 27

FIGURE 6: STRUCTURE OF EPIGALLOCATECHIN GALLATE (KANG ET AL., 2011) 29

FIGURE 7: GROWTH INHIBITION OF HL-60 CELLS AFTER INCUBATION WITH EA FOR 24, 48 AND 72H 39

FIGURE 8: CELL CYCLE DISTRIBUTION IN HL-60 CELLS AFTER INCUBATION WITH EA FOR 24H 43

FIGURE 9: CELL CYCLE DISTRIBUTION IN HL-60 CELLS AFTER INCUBATION WITH EA FOR 48H 44

FIGURE 10: *IN SITU* MEASUREMENT OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN HL-60 CELLS AFTER TREATMENT WITH EA 45

FIGURE 11: CONCENTRATION OF DNTP POOLS IN HL-60 CELLS AFTER TREATMENT WITH EA FOR 24H 46

FIGURE 12: GROWTH INHIBITION OF HL-60 CELLS AFTER INCUBATION WITH EGCG FOR 24, 48 AND 72H 47

FIGURE 13: CELL CYCLE DISTRIBUTION IN HL- 60 CELLS AFTER INCUBATION WITH EGCG FOR 24H 49

FIGURE 14: INDUCTION OF APOPTOSIS IN HL-60 CELLS AFTER INCUBATION WITH EGCG FOR 24H 51

FIGURE 15: INDUCTION OF APOPTOSIS IN HL-60 CELLS AFTER INCUBATION WITH EGCG FOR 48H 51

FIGURE 16: CELLULAR MORPHOLOGY OF HL-60 CELLS TREATED WITH EGCG FOR 24 AND 48H. CELLS WERE DOUBLE STAINED WITH HOECHST 33258 AND PROPIDIUM IODIDE 52

FIGURE 17: *IN SITU* MEASUREMENT OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN HL-60 CELLS AFTER TREATMENT WITH EGCG 53

FIGURE 18: CONCENTRATION OF DNTP POOLS IN HL-60 CELLS AFTER TREATMENT WITH EGCG FOR 24H 54

List of Tables

TABLE 1: COMBINATION EFFECTS OF EA AND ARA-C IN HL-60 CELLS EMPLOYING A SIMULTANEOUS GROWTH INHIBITION ASSAY **41**

TABLE 2: COMBINATION EFFECTS OF EA AND ARA-C IN HL-60 CELLS EMPLOYING A SEQUENTIAL GROWTH INHIBITION ASSAY **42**

TABLE 3: RADICAL SCAVENGING ACTIVITY OF EA AFTER INCUBATION FOR 15 MIN, GIVEN AS IC_{50} VALUE **44**

TABLE 4: COMBINATION EFFECTS OF EGCG AND ARA-C IN HL-60 CELLS EMPLOYING A SEQUENTIAL GROWTH INHIBITION ASSAY **48**

TABLE 5: RADICAL SCAVENGING ACTIVITY OF EGCG AFTER INCUBATION FOR 15 MIN, GIVEN AS IC_{50} VALUE **50**

ABBREVIATION INDEX

ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
Ara-C	cytarabine
ATCC	American type culture collection
ATO	Arsenic trioxide
ATRA	all-trans retinoic acid
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BMT	bone marrow transplantation
Cdc2	cell division control protein 2
CDK	cyclin-dependent kinases
CIP	CDK Interacting Protein
CKI	cyclin-dependent kinases inhibitors
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CR	complete remission
Cys	Cysteine
dATP	deoxyadenosine triphosphate
dFdC	Gemcitabine
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleotide acid
DNR	Daunorubicin
dNTP	deoxyribonucleotide triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl (free radical)
dTTP	deoxythymidine triphosphate
EA	Ellagic acid
EDTA	ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FCS	fetal calf serum
FLT3	Fms-like tyrosine kinase receptor 3
Fms	Feline McDonough Sarcoma
G_0, G_1, G_2	gap- or growth phases of the cell cycle
HAT	hydrogen atom transfer
HL-60	acute promyelocytic leukemia cell line
HO	Hoechst Dye 33258
HPLC	high performance liquid chromatography
HU	hydroxyurea
IC_{50}	concentration that causes 50% inhibition
IDA	Idaribicin
IKK	Inhibitor kappaB kinase
IL	interleukin
INK4A-C	Inhibitors of CDKs; class of cyclin-dependent kinases inhibitors

JNK	c-Jun N-terminal kinase pathway
KIP	Kinase Inhibitory Protein
M	molar
MAPK	mitogen-activated protein kinase
MDS	myelodysplastic syndrome
MeOH	methanol
mM	millimolar
M-phase	mitosis
NaCl	natrium chloride
NF-κB	nuclear factor-kappaB
p15,16, 18	members of INK4 family
p53	tumor suppressor protein; transcription factor
P53R2	homolog of R2
PBS	phosphate buffered saline
pH	pondus hydrogenii
PI	propidium iodide
PI3-K	phosphatidylinositol-3-kinase
PML	promyelocytic leukemia
R1	large α_2-homodimer of ribonucleotide reductase
R2	small β_2-homodimer of ribonucleotide reductase
RAR	retinoic acid nuclear receptors
RAR-α	retinoic acid receptor alpha
Rb	retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	cell culture medium (Roswell park memorial institute)
RR	ribonucleotide reductase
RXR	retinoid X receptor
SCT	stem-cell transplantation
SDS	sodium dodecyl sulphate
SET	single electron transfer
SFK	Src family kinase
S-phase	DNA-synthesis
TBS	tris buffered saline
TCA	trichloroacetic acid
TNF	tumor necrosis factor
TNO	Tri-n-octylamin
VEGF(R)	vascular endothelial growth factor (receptor)

1 Project aims

Cancer is a fatal disease that is present all over the world and is the leading cause of death after heart disease. Various organs of the body can be affected and more than 100 different types of cancer are known to this day. Genetic mutations are accountable for nearly all kinds of cancer (Hanahan & Weinberg, 2000).

As cancer is an intricate disease with numerous factors and processes being involved, it still remains a challenge to determine proper remedies. Existing cytostatic agents, especially chemotherapy, led to a considerably improved cure rate. Nevertheless, some synthetic drugs might bring about side effects when used in clinical trials. The formation of resistances uses to arise as well. That lack for appropriate treatment encourages researchers in finding alternative medications in order to fight cancer. Thus, focus has therefore been targeted on chemoprevention, comprising the use of food factors and supplementary substances, which might interfere with the development of this complex disease (Kang et al., 2011).

Polyphenols are a group of chemical substances, operating as secondary metabolites in plants. They are considered as aromatic compounds, comprising one or more hydroxyl group(s) attached to a construct of more than one phenol unit. According to their structure, they can be categorized into several classes. Polyphenols occur in a variety of human diets, including fruits, vegetables and beverages like tea. They are assumed to implement health-promoting properties by acting as anti-oxidants, anti-carcinogens and anti-inflammatories and are therefore linked to reduce the risk of developing cancer (Tsao, 2010).

Polyphenols belong to frequently consumed food components and beverages. They are supposed to show low toxicity, which gives them advantage over synthetic drugs. Consequently, polyphenols are suggested to operate as novel chemopreventive agents that exert fewer side effects (Kang et al., 2011).

Ellagic acid (EA), which is a dimer of gallic acid and belongs to the group of phenolic acids, and Epigallocatechin gallate (EGCG), which is a flavanol and is widely distributed in the leaves of green tea were studied with regard to their effect on the human HL-60 promyelocytic leukemia cell line. Leukemia is a disease or type of cancer that affects the blood and bone marrow.
As phenolic compounds are known to have a cytotoxic impact on the growth of cancer cells as well as an enhancing effect on cytostatic agents that are used in the treatment against cancer, it was hypothesized that EA and EGCG might exert these properties as well.

Studies on EGCG have revealed its cytotoxicity due to its anti-oxidative features. Furthermore, pro-oxidative effects, related to the induction of apoptosis in acute promyelocytic leukemia cancer cells were detected as well. Its role as a metal chelator has also been considered (Lambert & Elias, 2010). Synergistic interactions were observed when EA was combined with other polyphenols, leading to an inhibition of cell proliferation or cell cycle arrest in human acute lymphoblastic leukemia cells. This indicates that EA might possess an enhancing effect on existing cytostatics as well (Mertens-Talcott & Percival, 2005).

Both substances were reviewed for their cytotoxic effects employing a growth inhibition assay. Cell cycle distribution analysis was implemented using FACS. Hoechst/propidium iodide double staining was used to detect whether apoptosis was induced.
Ribonucleotide reductase (RR) is an enzyme that converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates and is therefore of high importance for DNA replication and the maintenance of deoxyribonucleoside triphosphate (dNTP) pool balance. The enzyme is highly up regulated in tumor cells, representing a suitable target in cancer therapy (Saiko et al., 2011).
To ascertain whether EA or EGCG inhibit the *in situ* activity of RR, DNA of cancer cells was incorporated with radiolabeled cytidine. A specific HPLC method was applied for the detection of dNTP pool imbalances.
Combination effects of both compounds together with Cytarabine (Ara-C), a clinically established cytostatic agent in the therapy of leukemia, were investigated by simultaneous and sequential growth inhibition assays.

The study sheds light on new biological activities of these natural compounds, offering promising prospects on further *in vivo* studies in order to establish new cancer therapies.

2 Introduction

2.1 Cancer

Cancer is a worldwide disease that is marked by changes in the genome, leading to severe mutations and chromosomal rearrangements. There exist a variety of about 100 different types of cancer located in diverse organs. Carcinogenesis in humans is considered to be a multistep process that is responsible for the transformation of normal cells into malignant cancer cells. These aberrant cells lack normal homeostasis and possess deficiencies in essential signaling pathways (Hanahan & Weinberg, 2000).

Mutations in cancer cells primarily affect tumor suppressor genes and proto-oncogenes that hence develop into oncogenes. Normally, tumor suppressor genes are responsible for a block in cell cycle progression, whereas proto-oncogenes function as activators for cell proliferation (Vermeulen et al., 2003 b). Therefore, gain of function mutations in oncogenes and mutations that generate tumor suppressor genes with loss of function result in a stimulated and uncontrolled growth of tumor cells.

It is suggested that most cancer types undergo six alterations that affect their cell physiology and consequently yield in malign cell growth. These hallmarks of cancer comprise self-sufficiency in proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, resistance to cell death, induction of angiogenesis and enabling of replicative immortality.
These capabilities achieved in tumorigenesis give cancer cells an advantage over normal cells and allow them to quickly proliferate and invade or metastasize to surrounding tissues (Hanahan & Weinberg, 2000).

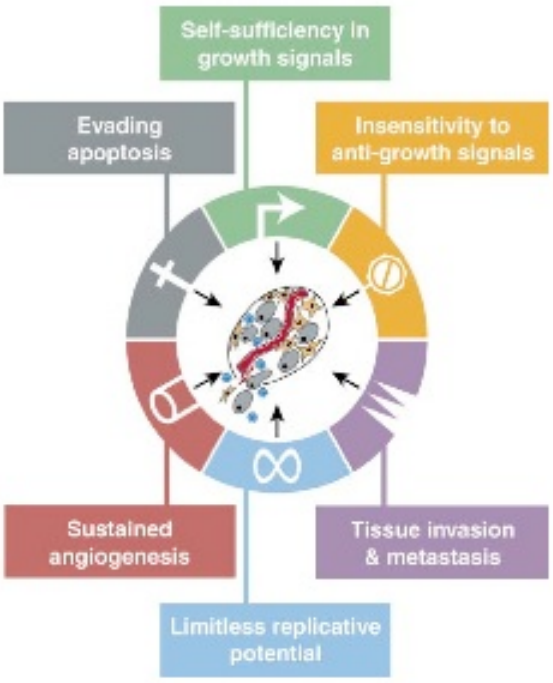

Figure 1: The hallmarks of cancer, comprising six abilities acquired by cancer cells (Hanahan & Weinberg, 2000)

During the last years, intensive research gave rise to the suggestion that two new hallmarks could possibly play a role in the pathogenesis of cancers. One involves the deregulation of cellular energetics, thus modulating a cell´s metabolism. As a result, this mechanism could support degenerated proliferation. The second ability contributing to neoplasia delineates the avoidance of immune destruction. Cancer cells thereby escape extinction by leucocytes or macrophages. As both capabilities are not fully validated to this day, they are considered as emerging hallmarks (Cavall et al., 2011; Hanahan & Weinberg, 2011).

2.2 Cell cycle and cancer

2.2.1 Cell division cycle

The cell division cycle depicts a fundamental process that enables mammalian cells to proliferate. It has four different phases used for growing, replicating DNA, and dividing into two daughter cells. In synthesis or S-phase, DNA is replicated. Mitosis or M-phase is the phase where chromosomes are separated, resulting in cell division. The two gap- or growth phases G1 and G2 follow M- and S-phase and are needed for cell growth and preparation for the next event in cell cycle progression, respectively (“SABiosciences, a QIAGEN company." ; Van den Heuvel, 2005). The quiescence or G0 phase is a state that harbors fully differentiated cells, which left G1 phase, but still have remaining tasks to fulfill within the organism (Garrett, 2001).

2.2.2 Cell cycle regulation

There exist several mechanisms to achieve an accurate cycling through the specific stages. The cyclin dependent kinase (CDK) family with its associated cyclin subunit illustrates important regulatory proteins. They are required at different phases and allow proper cell cycle transitions. CDK activity is regulated due to degradation through ubiquitin-mediated proteolysis, by CDK inhibitors (CKIs) or through reversible phosphorylation.

Different cyclin-CDK complexes occur at specific transition points. Cyclin D is associated to CDK4 or 6 when in G1 phase, cyclin E-CDK2 complex appears at the transition from G1 to S-phase, cyclin A is bound to CKD2 when in S-phase and cyclin A or B in a complex with CDK1 (also called cdc2) is involved at the entry into M-phase (Garrett, 2001; Meeran & Katiyar, 2008).

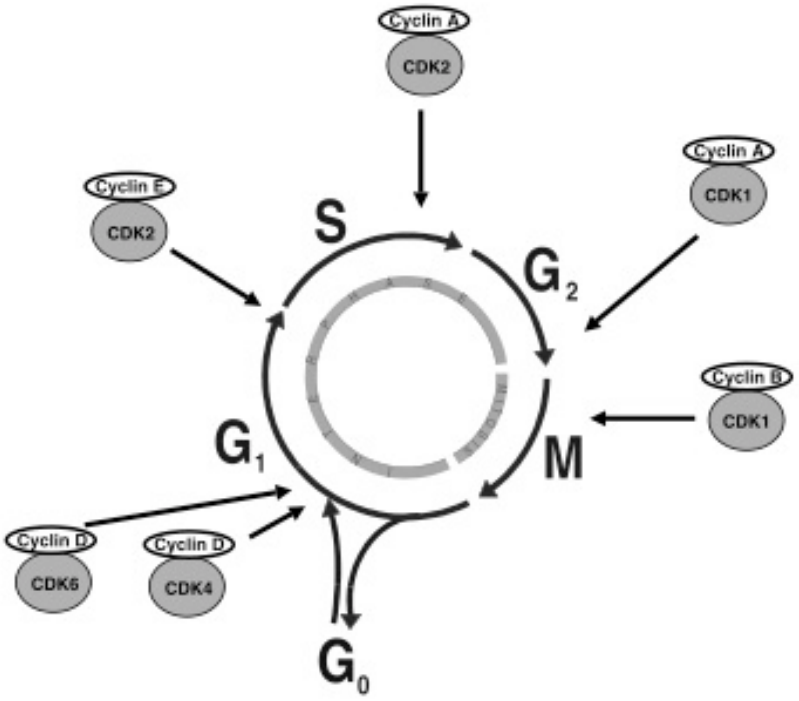

Figure 2: The different stages of cell cycle together with regulatory cyclin-CDK complexes (Vermeulen et al., 2003 b)

In order to ensure the correct chronology during each cell cycle, further sensor mechanisms, called checkpoints, occur. These checkpoints precisely control transitions through the cell cycle and ascertain that each phase is in appropriate condition before processing to the next stage. That proteins are essential for cell survival, as for example DNA damage causes an arrest in cell cycle progression and hence needs to be repaired properly (Garrett, 2001). Important checkpoint molecules comprise p53, retinoblastoma protein (Rb), p21, which belongs to the CIP/KIP (CDK Interacting Protein/ Kinase Inhibitory Protein) family, and different CKIs like p15 (INK4B), p16 (INK4A) and p18 (INK4C) (“SABiosciences, a QIAGEN company").

2.2.3 Cell cycle deregulation in cancer

A breakdown in the accurate regulation of the cell cycle can bring about serious consequences. Mutations in genes or defects in proteins involved in its regulation, like CKDs, cyclins and checkpoint proteins, lead to uncontrolled cell proliferation and thus contribute to the development of tumors (Vermeulen et al., 2003 a; Vermeulen et al., 2003 b).

2.3 Cell death and cancer

2.3.1 Apoptosis

The programmed cell death is of tremendous importance to all cells, as it depicts a natural protection mechanism against the development of cancer. Death signals activate proteases that lead to the induction of proteolysis, involving caspases, which ultimately results in apoptosis. The cells thereafter get disassembled and consumed by phagocytes. Several proteins play a role in the regulation of apoptosis, with some of them acting as inhibitors like Bcl-2 (B-cell lymphoma 2), while others function as pro-apoptotic regulators like Bax (Bcl-2-associated X protein). When Bax is not inhibited by anti-apoptotic factors like Bcl-2, it disrupts membrane integrity leading to the release of apoptotic proteins such as cytochrome *c*. As a result, a cascade of caspases is turned on and finally programmed cell death is initiated (Hanahan & Weinberg, 2011).

Tumor-suppressor protein p53 possesses a key function in the cell operating system. Depending on the severity of damage inflicted to the cell, p53 can either stop cell cycle progression or trigger apoptosis.

Deregulation in cancer

Tumor cells developed multiple ways to avoid apoptosis so as to gain uncontrolled cell growth and malignancy. They evolved different strategies to circumvent the variety of apoptosis-inducing signals. Several mechanisms have been revealed, including a decline in pro-apoptotic factors or an up-regulation of anti-apoptotic regulators. Furthermore, most cancers have mutations in tumor suppressor p53. DNA damage or increased levels of oncogenes account for imbalances in cell signaling and represent burdens that are accompanied with the induction of apoptosis (Hanahan & Weinberg, 2011; Vermeulen et al., 2003 a).

2.3.2 Necrosis

Necrosis has long been viewed as a form of cell death caused by organismic collapse or overexertion. Necrotic cells are denoted by a swollen appearance that soon after explodes. Their cell debris remains in the tissue environment, releasing pro-inflammatory signals that recruit immunocompetent cells in order to remove their contents.

However, there is rising evidence that necrosis might sometimes underlie genetic control and even participates in the development of neoplasia. This tumor promoting function could be due to the stimulation of immune inflammatory cells, as they are capable of encouraging angiogenesis and cell proliferation (Hanahan & Weinberg, 2011).

2.4 Ribonucleotide reductase

2.4.1 General information

Ribonucleotide reductase (RR) is an enzyme that is responsible for *de novo* DNA synthesis. It is able to reduce all four ribonucleoside di-(tri-) phosphates into 2´-

deoxyribonucleoside di-(tri-)phosphates. The 2´OH-group of the ribonucleotide is substituted by a hydrogen atom, resulting in the formation of a deoxyribonucleotide (Nordlund & Reichard, 2006). One of the four products, namely 5´-di-(tri-) phospho 2´-deoxyuridine has to be converted into thymidine by the enzyme thymidylate synthase so as to be used for DNA synthesis (Kolberg et al., 2004).

This transformation of RNA building blocks into DNA building blocks is catalyzed by a complex mechanism involving protein radicals. As RR is necessary for DNA replication and repair, all living organisms depend on this enzyme in order to survive and grow. There exist three different classes I, II and III, whereas all eukaryotic organisms posses a class I RR. An important distinction between the three classes of reductases lies in the generation of the protein radical by different metal cofactors.

RRs are able to maintain a balanced pool of all four deoxyribonucleoside triphosphates (dNTPs) in the cell because unequal production would increase the occurrence of mutations and would hence make the cell more prone to develop cancer (Reichard, 2010).

2.4.2 Classification of RRs

RRs are categorized into three main groups, based on their metal cofactors for catalytic activity as well as on their interaction with oxygen. They all have a conserved cysteine residue in common that is converted into a transient thiyl radical. This thiyl radical is assumed to be involved in the mechanism that is responsible for the abstraction of the 2´-hydrogen atom on the ribose ring of the substrate.

Class I enzymes are found in all eukaryotic organisms, ranging from yeast, plants and mammals. Some viruses and prokaryotes also belong to this group. All class I enzymes depend on oxygen (Kolberg et al., 2004). They contain two subgroups, Ia and Ib with class Ib only found in prokaryotes. The human RR belongs to subgroup Ia and is a tetrameric enzyme ($\alpha_2\beta_2$), which can further be divided into two dimeric subunits (R1 and R2). The larger homodimer $\alpha 2$ is called R1 and is

encoded by *nrdA* in class Ia. R1 comprises the catalytic side with substrate specificity as well as the allosteric site for regulation of the enzyme. R2 is the smaller homodimer β2, encoded by *nrdB*. This subunit contains a diiron-oxygen center (Fe-O-Fe), with a tyrosyl radical nearby. That tyrosyl radical is stabilized by the Fe-O-Fe center. The radical from subunit R2 is shuttled to a cysteine residue in R1, where it then generates the thiyl radical, which is needed for the activation of the substrate. In class Ia, electrons required for that complex radical transfer are provided from thioredoxin or glutaredoxin together with other redox-active small proteins (Nordlund & Reichard, 2006).

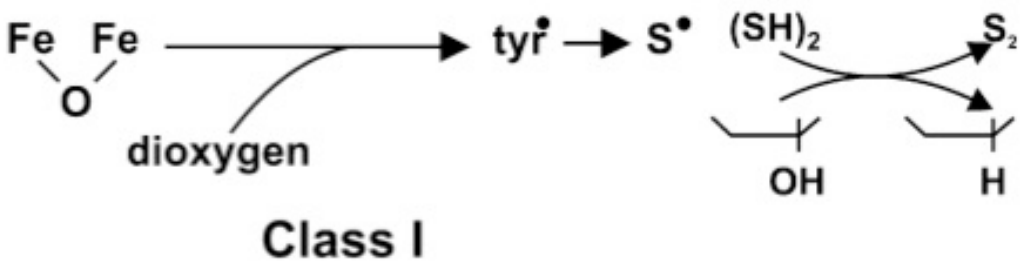

Figure 3: Radical generation in RR Class I. Subunit R2 produces the tyrosyl radical which then generates the thiyl radical on subunit R1 (Reichard, 2010).

Although the sequence homology of the three classes of RRs and their need for oxygen is very different, they have basic structural resemblances in common. These structural elements, comprising the catalytic domains, are the basic components for the substrate turnover. Hence, a similar reaction as well as allosteric mechanism is assumed. Due to those similitudes, RRs are supposed to have an evolutionary common ancestor, with class III being the nearest relative (Reichard, 2010).

2.4.3 Structure

The subunits of all three classes of RRs have similar structures. In particular, class I and II show a high accordance of this α/β topology.

R1 subunit protein of *E. coli*, which functions as a suitable model for that structure, has a core domain that builds up a 10-stranded α/β-barrel. The thiyl radical-forming cysteine residue (C439 in *E. coli*) is positioned in the centre of the barrel (Kolberg et al., 2004). The other two reducing cysteine residues (C225 and C462 in *E. coli*) are located on adjacent β-strands. Residues of the activity site, which are needed for the general activation of the enzyme, are allocated from the β-strands of the core barrel. The two effector binding sites or S-sites, regulating substrate specificity, are situated at the point of intersection of the α-dimer and are therefore compiled of residues from both dimer subunits. There exists also an ATP-cone domain at the N-terminal in the *E. coli* R1 protein, molded of a α-helical bundle, where effectors for the total enzyme activity can bind. The C-terminal region encompasses two cysteine residues (C754 and C759 in *E. coli*) needed for a complete substrate turnover cycle (Nordlund & Reichard, 2006).

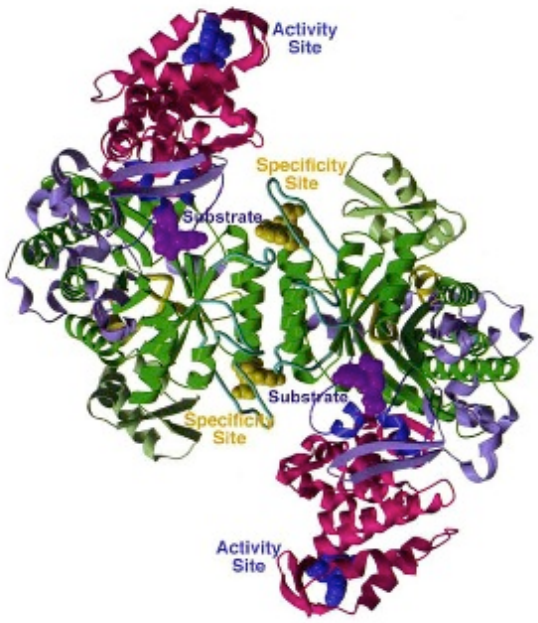

Figure 4: R1 subunit of *E. coli* with each monomer containing one binding site for the substrate, one allosteric specificity site and one allosteric activity site (Reichard, 2010).

The function of subunit R2 protein resides in generating and storing the initial radical needed for the reduction of ribonucleotides. The protein homodimers are primarily composed of α-helices. Within this α-bundle is the diiron center with the tyrosine residue close by. This residue (Y122 in *E. coli*) is responsible for the generation of the radical. As it is the diiron center that is accountable for generating the tyrosyl radical, it is supposed to hold a strong oxidation power (Kolberg et al., 2004).

2.4.4 Catalytic cycle, radical transfer & storage

As mentioned above, a remarkably long and complex radical transfer chain via specific amino acids is located between the tyrosyl radical buried in subunit R2 and the thiyl radical on R1. Since the distance between these subunits is estimated to be around 30 Å, the radical needs to travel a long-range route in order to facilitate the substrate turnover reaction at R1. Therefore, it is assumed that a reversible electron-transfer path alongside a chain of hydrogen-bonded

side residues transports this radical. The thiyl radical site C439 on R1 and the tyrosyl radical site Y122 in R2 are coupled to the surface of the enzyme by that pathway of hydrogen bonds (Nordlund & Reichard, 2006).

The diiron center of R2 subunit generates the tyrosyl radical. It is able to oxidize the tyrosine residue Y122 to a tyrosyl radical, thereafter reactivating molecular oxygen. That tyrosyl radical is supposed to be very stable. This is due to its isolated position within the α-helix pocket of the protein as well as to its poor interplay with the diiron-oxygen center (Kolberg et al., 2004).

2.4.5 Allosteric regulation

RRs possess two allosteric sites that make it possible to maintain a balanced pool of all four dNTPs. The activity site, or A-site, is responsible for the overall performance of the enzyme and determines whether RR is stimulated or inhibited, depending on the cellular concentrations of either ATP or dATP. The specificity site or so-called S-site determines which substrate will be reduced in order to produce deoxyribonucleotides (Nordlund & Reichard, 2006). This complex allosteric mechanism is of high importance to the cells. The correct amount of each dNTP pool needs to be provided, as they are involved in a huge number of intracellular regulation mechanisms and hence have to be controlled precisely (Reichard, 2010).

RRs are able to reduce all four ribonucleotides. Depending on the class, they either reduce 5´-di (or tri) phospho-adenosine, -guanosine, -cytidine and –uridine as substrates. When an allosteric effector binds to the so-called specificity site, this S-site determines which of the four substrates will be reduced at the catalytic site. Effectors are fully phosphorylated ATPs, dATPs, dTTPs, or dGTPs whereas dCTPs are assumed to have only a minimal effect. Substrates can either be ribonucleoside 5´-diphosphates, which is the case in class I and some class II enzymes, or ribonucleoside 5´-triphosphates in class II and III. When dATP binds to the S-site of class Ia enzymes, binding and reduction of CDPs and UDPs is promoted, whereas the binding of dTTPs inhibits their reduction but encourages

the reduction of GDPs. Binding of dGTP on the other hand inhibits the reduction of GDPs and stimulates the reduction of ADPs. Class I and II enzymes make no difference between ATP and dATP, therefore ATP is also able to initiate the reduction of UDPs and CDPs (Kolberg et al., 2004).

In all three classes, these substrate-binding sites have very similar structures. Effectors are assumed to induce conformational changes at the S-site, thereby allocating a signal to the catalytic site. So-called Loop 2 functions as a central structural element for the allosteric regulation of substrate binding. Both, effectors and substrates are capable of modulating the conformation of Loop 2. When an effector binds to the S-site, the modification of Loop 2´s conformation leads to its shift toward the related substrate. This indicates that there might occur cooperation between the catalytic and the specificity site (Nordlund & Reichard, 2006).

The general activity site, also called A-site, with its N-terminal ATP cone, operates as the second allosteric regulatory site. It is responsible for the on- or off-switch of the enzyme. The binding of ATP activates RRs, while binding of dATP exerts inhibiting effects. Consequently, dATP is supposed to exert stimulatory effects on the S-site together with inhibitory effects on the A-site. However, dATP is bound to the S-site with a significantly higher affinity in comparison to the A-site. This feedback regulation enables the cell to generate the balanced dNTP pool needed for DNA synthesis (Reichard, 2010).

2.4.6 RR and its occurrence during the cell cycle

During the synthesis (S-phase) of the cell cycle, DNA replication takes place. Hence, a large amount of dNTPs is a prerequisite for proliferating cells since sufficient supply of these building blocks is essential for cell survival. In the other phases, namely the growth phase G0, G1, mitosis (M-phase) and G2, the demand is much lower. As RR is closely related to the cell cycle, its expression level is highly up regulated in S-phase, whereas it decreases during the other phases.
Additionally, RR is supposed to play a role during DNA damage. Subunits R1 and

R2 are regulated in a different way, though both reaching highest levels of expression during S-phase. R1 is expressed during the whole cell cycle (Kolberg et al., 2004). R2 represents the rate limiting subunit of the enzyme and it is probably regulated transcriptionally as well as by enzyme degradation. During G1, binding of transcription factor E2F4 represses its transcription, while during M-phase, R2 is degraded due to ubiquitination and proteolysis (Nordlund & Reichard, 2006).

p53R2

Some studies reveal the existence of a second R2 subunit, p53R2, which can be observed in mouse and human cells. p53R2 is assumed to provide the cell with dNTPs after DNA damage, as normal subunit R2 is not functional in resting cells. This protein shows low levels of expression throughout the whole cell cycle and is presumably induced by tumor protein 53 (p53). Over 80% of human tumors have a mutated p53. These mutations can also affect pathways that are either involved in the regulation of p53 or that are regulated by the protein. This fact would lead to the expectation that cancer cells are incapable of activating p53R2 and consequently would die (Kolberg et al., 2004; Nordlund & Reichard, 2006). As studies showed that cells with mutated p53 could still induce p53R2, there has to exist another independent pathway that is able to activate that protein (Byun et al., 2002).

2.4.7 RR and cancer

RR is supposed to be up regulated in tumor cells as they are rapidly proliferating and hence have an increasing need of dNTPs for DNA synthesis. Therefore, specific inhibitors against this enzyme are used as chemotherapeutic treatments against cancer (Saiko et al., 2008). They are able to target preferably fast proliferating cancer cells, thus normal cells are only marginally affected (Horvath et al., 2005).

RR inhibitors can be applied alone or in combination with other drugs, like Ara-C. Agents that are commonly used are for example hydroxyurea (HU) or Gemcitabine (dFdC). Their mode of action lies in their ability to function as radical

scavengers or metal chelators. Subunit R2 of RR therefore offers a suitable target for inhibitors, as chelation of the diiron center or destruction of the essential tyrosyl radical can result in the destabilization of the enzyme. Thus, RR inhibitors lead to alterations and imbalances in dNTP pools as well (Saiko et al., 2008, 2011).

2.5 Leukemia

2.5.1 Introduction

Leukemia is a type of cancer affecting the blood and bone marrow. It is the most common form of cancer in children under 15 years of age. About two-thirds of all diagnosed cases of leukemia will die due to the disease (Deschler & Lübbert, 2006).
The most characteristic feature is an abnormal increased number of white blood cells, called leucocytes that can be found in the bloodstream of a person suffering from leukemia. Normal blood cells originate from the bone marrow as immature hematopoietic stem cells, which then develop into progenitor cells and further become different types of mature blood cells. In the majority of leukemia cases, abundant leukocytes appear in their immature and genetically unstable form that no longer proliferate and differentiate in a normal way. By this means they displace normal blood cells (Misaghian et al., 2009).

Leukemias are categorized into two different kinds, depending on their origin of leukocytes as well as on their mode of progression. Cells of myeloid lineages comprise erythrocytes, platelets and granulocytes, with the latter deriving from a myeloblast. Natural killer cells, B- and T- lymphocytes all descend from a lymphoblast and belong to lymphoid origin. Granulocytes, natural killer cells, B- and T-cells are assigned to the group of leucocytes.
Furthermore, leukemias can be divided into acute and chronic leukemia. Chronic leukemias (CML or CLL) possess mature leucocytes but show highly increased lymphocyte counts in the blood and bone marrow. It represents an insidious

disease with patients living with it for years without having any obvious symptoms.
In contrast, acute leukemias (AML or ALL) are characterized by the occurrence of immature progenitor cells that either show lineage differentiation or not and also emerge in abnormally high numbers. They arise very quickly and include early symptoms like infections, fever, fatigue or easy bleeding (Misaghian et al., 2009; "Comprehensive Cancer Information - National Cancer Institute").

Although the exact reason for developing leukemia is unclear and an outbreak also varies between the different types of this cancer, there are some risk factors that might play a role in the onset of that disease. These factors include congenital genetic defects or disorders, exposure to higher levels of radiation or benzene, or preceding chemotherapy.

Therapy varies among the different forms of leukemia as well as the age of the patient. The main goal is to get rid of leukemia cells from the body in order to achieve complete remission (CR). To prevent a relapse, consolidation or maintenance therapy is administrated. Treatment includes the induction of chemotherapy, occasional radiation therapy and some patients undergo stem cell transplantation (SCT) as well (“Comprehensive Cancer Information - National Cancer Institute,”).

2.5.2 Acute myeloid leukemia

AML represents the second most frequent form of leukemia after chronic lymphocytic leukemia (CLL), counting for approximately 25% of all cases of leukemia. That disease can affect all age groups, although there is a much higher incidence of the elderly population developing AML than young people (Zuo et al., 2009). The average age of getting AML is around 60 years and there exists a slightly bigger incidence for males to develop this form of leukemia (Deschler & Lübbert, 2006).

Acute myeloid leukemia (AML) is a disorder hallmarked by the rapid clonal

overproduction of immature hematopoetic progenitor cells called blasts. As these cells lack normal differentiation, hematopoiesis does not work properly any longer. If left untreated, that severe form of leukemia can lead to death within several weeks or months. Factors leading to an outbreak include the patient's age, its karyotype, genetic mutations or alterations characteristic for AML as well as existing co-morbidities like the myelodysplastic syndrome (MDS), together with others (Shipley & Butera, 2009).

The three most common cytogenetic abnormalities that can be ascribed to AML comprise reciprocal translocations, namely t(8;21)(q22; q22), the translocation or inversion of chromosome 16 t(16;16)(p13.1;q22) or inv(16) and translocation t(15;17)(q22;q12), which is typically found in acute promyelocytic leukemia (PML). They all underlie a distinctive morphology and clinical characteristics (Zuo et al., 2009).

Treatment

Standard treatment for AML includes chemotherapy using different anthracyclines, with Daunorubicin (DNR) being the most established one, and the administration of the cytostatic agent cytabine (Ara-C). Ara-C is a deoxycytidine analogue that is non-productively incorporated into nascent DNA. Depending on age and gene expression profiles, the impact of standard therapy differs between patients suffering from AML. As elderly persons are more prone to develop co-morbidities and are also more susceptible to have unfavorable cytogenetics due to their age, they usually show a poor response to treatment when compared with younger people. Therefore, remission rates vary from 50% to 85% in the population and the mortality rate is assumed to rise with increasing age.
Post remission therapy can comprise additional chemotherapy, bone marrow transplantation (BMT) or stem cell transplantation, autologous or allogeneic, respectively. Consolidation chemotherapy encompasses highly dosed Ara-C administration (Shipley & Butera, 2009).

Unfortunately, there exist deviations of Ara-C effectiveness between AML patients. This might be due to a different metabolism, age and co-morbidities, the

potency of drug uptake, as well as genetics (Cai et al., 2008).

Research on new therapeutic agents performed over the last years give promising prospects on novel therapies for AML. Signaling pathways and cell surface molecules in leukemic cells depict a target for new medicinal remedies like small molecule inhibitors and cytotoxic antibodies. Substances that show enhancing effects on already existing drugs are also in the field of investigation.
Fms (Feline McDonough Sarcoma)-like tyrosine kinase receptor 3 (FLT3) is expressed in hematopoietic progenitor cells and plays a role in cell proliferation and differentiation. About 30% of adult AML patients display mutations in FLT3, leading to a constitutional activation of the tyrosine kinase activity. Therefore, novel FLT3 tyrosine kinase inhibitors are under investigation. Transcriptional modulators, multidrug resistance modulators, farnesyl transferase inhibitors and others are surveyed as well (Shipley & Butera, 2009; Zuo et al., 2009).

2.5.3 Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) constitutes a rare subtype of AML. It is usually diagnosed in the second decade of life and early adulthood. Patients with APL are susceptible to have a coagulopathy at diagnosis and especially in children the presence of a hyperleukocytosis can be observed. Most cases of APL have a reciprocal translocation involving the promyelocytic leukemia (PML) gene on chromosome 15 and the retinoic acid receptor alpha (RARα) on chromosome 17 (Yoo, 2011). The fusion transcript PML-RARα can occur as three different isoforms. Morphologically, 75% of APL cells are hypergranular promyelocytes that have lobed nuclei, abundant azurophilic granules and numerous Auer rods. Auer rods are small and bacillary azurophilic granular particles that are found in the cytoplasm of leukemic blasts. Microgranular promyelocytes account for 25% of cases, with cells having bilobed nuclei and nearly no Auer rods (Zuo et al., 2009).

RARα on chromosome 17 belongs to the family of retinoic acid nuclear receptors (RAR), which are classified as transcription factors and regulate gene expression

in the presence of a ligand. Especially hematopoietic cells express these RA receptors. RAR and the retinoid X receptor (RXR) form heterodimers that repress transcription by recruiting co-repressors when the ligand RA is absent. Binding of RA causes a conformational change, activating gene transcription and hence differentiation of promyelocytes (Yoo, 2011).
The fusion protein PML-RARα leads to a stronger interaction of RAR with the co-repressor complex and results in the suppression of RARα regulated genes even in the attendance of ligand RA, causing a block in granulocyte differentiation. Only pharmacological doses of RA can interfere in that transcriptional blockade by transforming PML-RARα to an activator that restores normal promyelocyte differentiation (Zuo et al., 2009).

The standard treatment for patients with APL is the combination therapy of all-trans retinoic acid (ATRA) and chemotherapy with anthracyclines like Idaribicin (IDA) or Daunorubicin (DNR). As promyelocytic blasts are highly sensitive to chemotherapy, complete remission rates are between 90- and 95%. Arsenic trioxide (ATO) is another efficient active substance used in APL therapy. Its induction at low concentrations induces partial differentiation of APL cells, whereas higher concentrations can lead to apoptosis of blasts. It is still being considered whether ATO in combination with ATRA and chemotherapy can act as new front-line therapy for patients with newly diagnosed APL (Yoo, 2011).

2.6 Polyphenols

2.6.1 General information

Polyphenols represent a widely distributed group of chemical substances in the plant kingdom. Plants produce polyphenols as secondary metabolites in order to protect themselves from predators (Tsao, 2010). Polyphenols are derived from the amino acids phenylalanine and tyrosine and they contain more than one phenol unit per molecule. These phenols are bound by one or more hydroxyl group(s), and are considered as aromatic compounds (Kang et al., 2011). In plants, polyphenols mostly occur as glycosides, containing distinctive sugar units at different positions on their chemical construct.

According to the number of hydroxylated aromatic rings, as well as the functional groups these structures comprise, phenolic compounds can be divided into several classes (Gamaro et al., 2011). There exist more than 8000 of these natural products and the sub-groups polyphenols encompass are very diverse. Due to the vast variety of polyphenols in plants, they are categorized according to their chemical structure, their origin and their biological function (Tsao, 2010).

Polyphenols can be divided into flavonoids, chalcones, phenolic acids, stilbenes and phenolic alcohols/lignans. Best-studied are flavonoids, including flavonols, flavones, flavanones, isoflavones, flavanols, anthocyanidins and catechins.

Polyphenols are considered to play an important part in human diet and are found in natural foods like berries and grapes/wine, chocolate/cocoa, tea, coffee, soybeans and many other fruits, vegetables and whole grains. These dietary polyphenols are also suggested to have an essential impact on human health (Kang et al., 2011).

2.6.2 Polyphenols and Human Health

Dietary polyphenols have gained huge interest and enormous attention among food scientists and nutritionists due to their beneficial effects on human health throughout the last years. Researchers found out that these compounds might

have anti-oxidative, anti-carcinogenic and anti-inflammatory properties and therefore could play a role in the prevention of many diseases. Special focus is directed towards degenerative diseases, like for example cardiovascular- and neurodegenerative diseases as well as cancer (Weng & Yen, 2012).

Cancer still causes thousands of deaths worldwide nowadays, representing the second most common cause of death after heart disease. As it embodies a very complex illness, with lots of dynamic processes and factors involved, it is very hard and challenging to cure that disease to this day.

The lack of finding proper treatment options has led to increasing interest in the discovery of alternative strategies and approaches in cancer research. Intensive focus has therefore been put on chemoprevention, which includes the use of many food factors and supplementary substances that interfere with the development of cancer. In carcinogenesis, oxidative stress and modifications of cell signaling pathways caused by reactive oxygen species (ROS) play a significant role. ROS can lead to dysfunctional cell growth or cell death, which further can give rise to cancer formation and inflammation (Kang et al., 2011).

Polyphenols are supposed to be strong antioxidants that act as a defense mechanism against the appearance of ROS. They are also assumed to be involved in the modulation of cell signaling pathways.
As fruits, vegetables and whole grains are rich in polyphenolic compounds, they are linked to a lower risk of cancer development.

Therefore, a high consumption of such dietary polyphenols may help to lower the risk of developing these chronic diseases including cancer and many other degenerative diseases (Tsao, 2010).

2.6.3 Biological roles of Polyphenols

Anti-oxidative effects

Reactive oxygen species (ROS) are produced in aerobic metabolic pathways in the body, causing oxidative stress that is also linked to tumor formation. ROS include superoxide (O_2^-), hydroxyl (OH^-), hydroperoxyl (HOO^-), alkoxyl (RO^-) and peroxyl (ROO^-) radicals (Kang et al., 2011).
Polyphenols are assumed to be strong antioxidants. They are able to neutralize free radicals by either offering a hydrogen atom or an electron. This is possible due to their hydroxyl groups, which are important parts of their highly conjugated construct. Polyphenols can therefore decrease or suppress the formation of free radicals by deactivating active species of radicals or their precursors, or even by inhibiting their formation (Tsao, 2010).

Apart from their role in free radical scavenging, studies suggest that polyphenols can also be implied in direct regulation of enzymes that are involved in oxidative stress. Some of these polyphenolic compounds can increase the expression and the activity of anti-oxidative enzymes, like for example catalase, superoxide dismutase, and glutathione reductase as well as glutathione peroxidase. In addition, some polyphenols are able to reduce enzymes that exert anti-oxidative effects, like xanthine oxidase activity or NADPH oxidase activity. They therefore have a supportive role as well, operating as co-antioxidant (Kang et al., 2011).

Moreover, polyphenols are also thought to act as metal chelators, especially for transition metals like iron (Fe^{2+}). Chelation can prevent oxidation, which is caused by hydroxyl radicals. It can also reduce the rate of Fenton reaction, which is the main source of ROS in cells (Tsao, 2010).

Other effects

The inflammatory response is also correlated to oxidative stress. Several inflammatory gene products, like tumor necrosis factor (TNF), interleukins (IL), chemokines, as well as vascular endothelial growth factor (VEGF), play a crucial

role in the suppression of proliferation, angiogenesis and apoptosis. As inflammation is strongly associated with tumor promotion, polyphenols and their effective anti-oxidative activities might also have chemopreventive effects in several stages of tumor formation. That might be due to multiple factors and mechanisms that could be mediated by anti-oxidants (Kang et al., 2011).

Some of the previously mentioned direct and indirect anti-oxidative activities of polyphenols led to an emerging interest among scientists about their potential roles at cellular levels. Researchers take into consideration that these polyphenolic compounds as well as their metabolites might act as modulators in some cell signaling pathways, in addition to their electron-donating anti-oxidative activities.

Thus, a better understanding of the molecular mechanisms of polyphenols and the involved cellular pathways might lead to new insights concerning chemoprevention (Tsao, 2010).

2.6.4 Polyphenols and signaling cascades

Numerous signaling pathways are involved in cancer cells, varying between different types of cancer. Such signaling cascades have very important roles in tumor formation and can affect apoptosis, proliferation, cell migration, invasion and metastasis (Hanahan & Weinberg, 2000).

There is also evidence that cell cycle signals and checkpoint proteins illustrate key players in carcinogenesis. Genes encoding enzymes like cyclins or cyclin-dependant kinases (CDKs) are often mutated in cancer cells (Vermeulen et al., 2003 b). Such mutations or an aberrant regulation of gene expression can also have its origins in epigenetics. The post-translational modifications of histone tails, namely methylation, phosphorylation or acetylation, can be influenced by environmental factors, such as nutrients (Kang et al., 2011).
When receptors, like for example the epidermal growth factor receptor (EGFR) or the vascular endothelial growth factor receptor (VEGFR) are activated by

extracellular signals, they covert such stimuli into intracellular signals (Hanahan & Weinberg, 2011). These signals activate kinases, in most cases Src family kinases (SFKs), which then transfer the signals to different cellular pathways. The EGFR and the VEGF receptor both stimulate signal transduction cascades, like mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) pathways. Thus, they are also considered to promote tumor growth. SFKs are non-receptor tyrosine kinases and their activities are correlated with malignancy in different types of cancer. MAPKs regulate functions ranging from proliferation, differentiation and apoptosis. As these features all have an influence on tumor development, MAPK pathways are also considered to play a role in tumor formation. The PI3-K signaling pathway promotes growth and cell survival and is activated in different cancer types as well. In the majority of tumors, the extracellular signal-regulated kinase (ERK) pathway, c-Jun N-terminal kinase pathway (JNK) and Inhibitor kappaB kinase/nuclear factor-kappaB (IKK/NF-κB) signaling pathway are all deregulated or modulated, leading to tumor formation and progression (Kang et al., 2011).

Many existing synthetic drugs have high specificities and target only special proteins. However, they can lead to side effects when used in clinical trials and also might cause the formation of resistances. In contrast, polyphenols show low toxicity and exert inhibiting effects in multiple signaling cascades (Weng & Yen, 2012). They are present in many food components and are consumed over a long passage of time, which gives them an advantage over synthetic drugs. As mentioned before, nutrients can also be involved in epigenetic programming and can therefore bear positive effects on cancer cells.

Hence, polyphenols are suggested to function as novel chemopreventive agents with fewer side effects (Kang et al., 2011).

2.7 Ellagic acid

Ellagic acid (EA) is a dimer of gallic acid, which belongs to the group of phenolic acids (Madlener et al., 2007). Phenolic acids are polyphenolic compounds that can be separated into two types, namely benzoic acid and cinnamic acid derivates. Classification depends on their C6-C1 and C6-C3 backbones. Gallic acid belongs to the group of benzoic acids. They can occur as free phenolic acids or in their bound form. EA appears in its free form, whereas it originally derives from ellagitannins, which refer to the group of hydrolysable tannins (Tsao, 2010).

EA is widely distributed in fruits and nuts, like strawberries, raspberries, grapes, pomegranates and walnuts. The daily intake of EA per person through vegetables and fruits amounts approximately 6 mg (Priyadarsini et al., 2002).

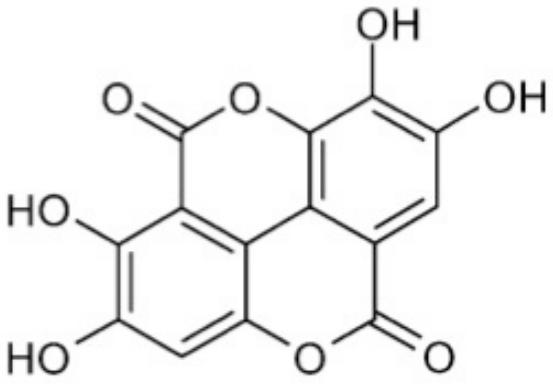

Figure 5: Structure of ellagic acid (Mertens-Talcott & Percival, 2005)

EA is supposed to have health supporting effects. It has been shown to exert anti-inflammatory, anti-mutagenic, anti-carcinogenic and anti-oxidative activities. Special focus is ascribed toward its role as an antioxidant. EA is assumed to scavenge ROS or inhibit their production. For this purpose, its hydroxy groups as

well as the lactone rings represent important structural features (Priyadarsini et al., 2002).

BA Narayanan et al. reported in 1999 that important anti-carcinogenic mechanisms of EA include the inhibition of cell growth, arrest in G0/G1-phase and S-phase of the cell cycle as well as the induction of apoptosis in colorectal adenocarcinoma and cervical epithelial carcinoma cells (Narayanan et al., 1999). On the other hand, Mertens-Talcott et al. detected no significant changes in cell numbers of human acute lymphoblastic leukemia cells and EA also had no major impact on the cell cycle distribution when compared to the control. In combination experiments with other polyphenols like quercetin or resveratrol, an inhibition of the cell proliferation, G0/G1-phase and S-phase arrests as well as the occurrence of apoptotic cell death could be perceived, indicating synergistic interactions of combined polyphenols (Mertens-Talcott & Percival, 2005).

2.8 Epigallocatechin gallate (EGCG)

EGCG is a flavanol or flavan-3-ol, which represent a subgroup of flavonoids. They are termed catechins and possess two chiral centers, and isomers can occur as catechin and epicatechin. Flavanols can be found in plants and fruits, mainly in blueberries, apples and in the skins of grapes. They also occur in cacao beans and tealeaves (Tsao, 2010).

EGCG is the most abundant polyphenol in green tea. Green tea as well as black tea and oolong tea derive from *Camellia sinensis.* Tea is one of the most popular beverages and is consumed all over the world (Chen & Dou, 2008). Green tea is supposed to contribute to a better health and it is attributed to exert cancer-preventive effects. As green tea with its most efficacious component EGCG is not assumed to have any adverse or toxic effects on human health, it is considered a promising therapeutic agent (Huo et al., 2008).

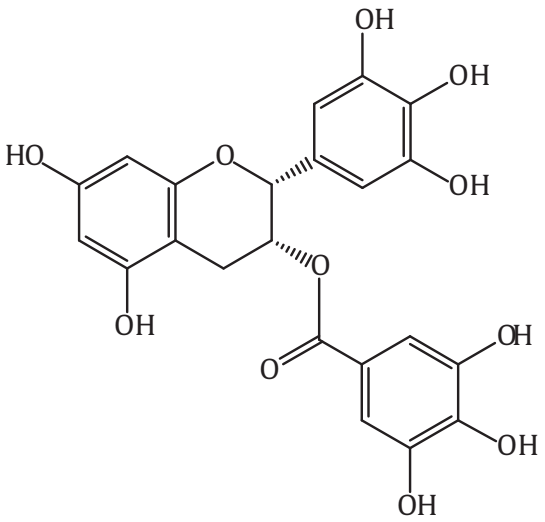

Figure 6: Structure of epigallocatechin gallate (Kang et al., 2011)

Free radicals produced by oxidation in naturally occurring metabolic pathways can lead to protein and DNA damages in the body. Commonly, antioxidant enzymes quench occurring ROS. Some diseases like heart disease, diabetes and cancer are exposed to a high level of oxidative stress, accompanied by damaged tissues (Chen & Dou, 2008).
EGCG is supposed to act as an important antioxidant. Mechanisms to get rid of ROS generally include hydrogen atom transfer (HAT) or single electron transfer (SET). The hydroxyl groups used are located on B- and D- rings of EGCG. These antioxidant effects were observed *in vitro* but some studies have demonstrated them to occur *in vivo* as well.

EGCG is able to function as a metal chelator. Transitions metals like iron or copper are competent catalysts at inducing phenolic oxidation, preferably within an alkali pH. They generate ROS in the form of superoxide O_2^{-}• or its protonated hydroperoxyl radical HO_2•, which is subsequently reduced to hydrogen peroxide H_2O_2. Catechol oxidation is always linked with metal reduction, like for e.g. Fe^{3+} -> Fe^{2+}. Therefore, transitions metals are supposed to be responsible for some pro-oxidant activities of polyphenols in green tea.
Several studies have revealed a deeper insight into the pro-oxidative effects of EGCG. As green tea polyphenols tend to undergo auto-oxidation at distinctive

conditions, including their own concentration, the pH of a system, the presence of oxygen and others, they thereby produce ROS. This can further lead to the induction of apoptosis *in vitro* and *in vivo* (Lambert & Elias, 2010).

Nakazato et al. showed that EGCG leads to a cell cycle arrest in G0/G1-phase in acute promyelocytic leukemia cells, which is followed by apoptosis. Although the exact mechanism of how EGCG induces apoptosis is not fully annotated, it is supposed that mitochondrial changes conduce to the release of cytochrome *c* into the cytoplasm und subsequently activate caspases. Elevated levels of ROS, associated with the induction of apoptosis, were observed as well (Nakazato et al., 2005).

Another hypothesis indicates that EGCG-induced oxidative stress may contribute to an enhanced activation of endogenous antioxidants like superoxide dismutase (SOD) or catalase, consequently leading to a decrease of free radicals (Lambert & Elias, 2010).

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and supplies

Cytarabine (Ara-C) and solvent DMSO were received from Sigma-Aldrich GmbH, Vienna, Austria and were of highest purity available. **Ellagic acid** (EA) was obtained from Sigma-Aldrich GmbH, Vienna, Austria and **Epigallocatechin gallate** (EGCG) was obtained from Sigma-Aldrich GmbH as well.
All other chemicals and reagents used were commercially available and of highest purity.
The following reagents, chemicals and materials were purchased as described below:

Acetonitrile	J.T. Baker
Ammoniumdihydrogenphosphate	VWR Merck, Vienna, Austria
Adenosine triphosphate	SIGMA, St. Louis, MO
Cellpack puffer	Mueller, Vienna, Austria
Chloroform	SIGMA, St. Louis, MO
Cytidine-2-14-C (56 Ci/mmol)	SIGMA, St. Louis, MO
Cytidine triphosphate	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
Hoechst dye 33258	SIGMA, St. Louis, MO
L-Rhamnose	SIGMA, St. Louis, MO
Methylamine	SIGMA, St. Louis, MO

Phenol	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
Thymidine triphosphate	SIGMA, St. Louis, MO
Trichloroacetic acid	SIGMA, St. Louis, MO
Tri-n-octylamine	SIGMA, St. Louis, MO
TRIS/EDTA buffer	SIGMA, St. Louis, MO
Uridine triphosphate	SIGMA, St. Louis, MO

3.1.2 Devices

CC-110 microcellcounter	SYSMEX, Kobe, Japan
Centrifuge 5415R	Eppendorf
Certoclav CV/CV-EL Autoclav	Certoclav, Vienna, Austria
Cytoperm 2 incubator	Heraeus, Vienna, Austria
Hettich Rotanta Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany
Laminar air flow	Dipl. Ing. W. Ehret GmbH, Emmendingen, Germany
Magnetic Stirrer	Framo, Eisenbach, Germany
Mettler PJ 300/AT 250 scales	Mettler, Vienna, Austria
Olympus IMT-2 Inverse	Olympus, Vienna, Austria
PH-Meter	Metrohm, Switzerland
Water Bath	Gesellschaft für Labortechnik GmbH, 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).

Dulbecco´s Modified Eagle Medium High Glucose
RPMI 1640 Medium with L-Glutamine
RPMI 1640 Medium with GLUTAMAX
Fetal Calf Serum (FCS), Heat Inactivated
Penicillin/Streptomycin 10.000 U/ml solution
L-Glutamine 200mM solution
Trypsin-EDTA 0.25% solution
Sodium Pyruvate 100mM

3.1.4 Cell lines

The human **HL-60** promyelocytic leukemia cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The cell line was grown in RPMI 1640 medium, which was supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-Glutamine, and 1% Penicillin-streptomycin. The **AsPC-1 and BxPC-3** human pancreatic cancer cells were grown in RPMI 1640 Medium with GLUTAMAX that was supplemented with 10% heat inactivated fetal calf serum (FCS), 1% Sodium Pyruvat and 1% Penicillin-streptomycin. **PANC-1** human pancreatic cancer cells were grown in Dulbecco's Modified Eagle Medium High Glucose supplemented with 10% heat inactivated fetal calf serum (FCS), 2% L-Glutamine, 1% Penicillin-Streptomycin and 1% Sodium Pyruvate. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2 using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria).
The attaching pancreatic cancer cells were grown in a monolayer culture using 25cm^2 tissue culture flasks and were periodically detached from the flask surface using 0.25% trypsin ethylene–diamine tetraacetic acid (trypsin–EDTA) solution.
All media and supplements were purchased from Life Technologies (Paisley, Scotland, UK). 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

Logarithmically growing HL-60 cells were seeded in T-25 cm^2 tissue culture flasks at a concentration of 0.1 x 10^6 per ml. The tumor cells were incubated with increasing concentrations of EA or EGCG (10, 20, 30, 40 and 50μM) at 37°C under cell culture conditions. Stock solutions were diluted in DMSO. After 24, 48 and 72h, cell counts and IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) were determined using the microcellcounter CC-110. Results were calculated as number of viable cells.

Simultaneous growth inhibition assay using EA or EGCG and Ara-C

To investigate the combination effects of EA with Ara-C, HL-60 cells (0.15 x 10^6 per ml) were simultaneously incubated with various concentrations of EA (10, 20 and 30μM) and Ara-C (5, 10 and 20nM) at 37°C under cell culture conditions. After 24, 48 and 72h, cells were counted using the microcellcounter CC-110.

Sequential growth inhibition assay using EA or EGCG and Ara-C

In the sequential growth inhibition assay, HL-60 cells (0.15 x 10^6 per ml) were first treated with EA (10, 20 and 30μM) or EGCG (15, 20 and 25μM) and incubated for 24h at 37°C under cell culture conditions. Afterwards, cell count was determined, EA or EGCG was washed out, and cells were resuspended in complete RPMI medium. Next, cells were exposed to different concentrations of Ara-C (5, 10 and 20nM) and incubated for another 24 and 48h. After these periods, cells were counted using the microcellcounter CC-110.

3.2.2 Cell cycle distribution analysis

HL-60 cells (0.4 x 10^6 per ml) were seeded in T-25 cm^2 tissue culture flasks. They were incubated with increasing concentrations of EA (60, 90 and 120μM for 24h and 30, 45 and 60μM for 48h) and EGCG (10, 20, 30, 40 and 50μM) for 24h at 37°C under cell culture condition. After 24 and 48h, cells were harvested and suspended in 5 ml cold PBS. Subsequently, the cells were centrifuged at 1000 rpm for 5 min, resuspended and fixed in 1ml cold 70 % EtOH (ethanol) for 30 min at 4°C. After two washing steps in cold PBS, 50 μg/ml RNAse A and 50 μg/ml propidium iodide were added to the cells and incubated at 4°C for 60min before measurement. Cells were then analyzed using 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).

3.2.3 DPPH• radical scavenging activity assay

The radical scavenging activity of EA and EGCG was determined using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). DPPH• in its radical form absorbs at a wavelength of 515nm whereas when reduced by an antioxidant or radical species, its absorption decreases. The radical scavenging activity for each compound was compared to that of α-Tocopherol and ascorbic acid. The reaction was initiated by adding different concentrations (1, 5, 10, 25 and 50μM) of EA, EGCG, α-Tocopherol and ascorbic acid to 200μl of 0.1mM DPPH• in MeOH (methanol). The DPPH radical has a purple staining which can be ascribed to the unpaired electron on the nitrogen atom. This stain turns into yellow when the radical binds to a hydrogen atom of a radical scavenger, giving rise to the reduced form of DPPH (2,2-Diphenyl-1-picrylhydrazin, DPPH-H). The bleaching was followed in a 96-well microtiter plate using a Wallac 1420 Victor 2 Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences). Absorbance was recorded for up to 15 min, although steady states of reaction were reached within 5 min in most cases. Reference controls contained 0.1 mM DPPH• in 200μl methanol.

3.2.4 Hoechst dye 33258 and propidium iodide double staining

HL-60 cells (0.2 x 10^6 per ml) were seeded in T-25 cm^2 tissue culture flasks with increasing concentrations of EA (60, 90 and 120μM for 24h and 30, 45 and 60μM for 48h) and EGCG (10, 20, 30, 40 and 50μM) for 24 and 48h at 37°C under cell culture conditions. After 24 and 48h, cells were transferred into a 96-well microtiter plate. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) were added to a final concentration of 5 μg/ml of HO and 2 μg/ml of PI, respectively. Afterwards, HL-60 cells were incubated for 60 to 90 min at 37°C. Cells were then examined on a Nikon Eclipse TE-300 Inverted Epi-Fluorescence Microscope (Nikon, Tokyo, Japan) equipped with a Nikon DS-5M-L1 Digital Sight Camera System including appropriate filters for HO and PI. This method, together with fluorescence microscopy, enables to distinguish between early apoptosis, late apoptosis and necrosis and also provides morphological information. The HO dye stains the nuclei of all cells and therefore indicates all cellular changes associated with apoptosis, like nuclear fragmentation or chromatin condensation. PI uptake portends late apoptotic as well as necrotic cells that have lost membrane integrity, as viable and early apoptotic cells cannot incorporate PI. The selective uptake of the two dyes in addition to the morphologies of nuclei allows distinguishing between the induction of apoptosis and necrosis in intact cultures. Cells were rated according to the integrity of their cell membranes, due to the staining of PI, and their morphology. HL-60 cells were then counted under the microscope and the number of apoptotic cells was given as percentage value.

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

To analyze the effect of EA or EGCG treatment on the activity of DNA synthesis, an assay was performed as previously described by Szekeres et al. Logarithmically growing HL-60 cells (0.8 x 10^6 per ml) were incubated with 60μM of EA for 48h and with 20μM of EGCG for 24h at 37°C under cell culture conditions. After the incubation period, cells were counted and afterwards pulsed with ^{14}C-labeled cytidine (3.125μl per 5ml medium) for 30 minutes at 37°C.

Radiolabeled cytidine has to be reduced by RR in order to be incorporated into the DNA of HL-60 cells. Subsequently, cells were collected by centrifugation (1200 rpm for 5 min) and washed with PBS. Total DNA was isolated from 5 x 10^6 cells and was purified by Phenol-Chloroform-Isoamylalcohol extraction. Specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (PerkinElmer, Boston, MA, USA) whose read out was normalized by a Hitachi U-2000 Double Beam Spectrometer to ensure equal amounts and purity of DNA.

3.2.6 Determination of deoxyribonucleoside triphosphates (dNTPs)

The extraction of cellular dNTPs was performed according to a method described by Garrett and Santi. HL-60 cells were seeded in 175 cm^2 tissue culture flasks and incubated with increasing concentrations of EA (60, 90 and 120µM) or EGCG (10, 20, 30 and 40µM) for 24 h. After the incubation period, cell count was determined and 1.5 x 10^8 cells were separated for dNTP extraction. All extraction steps were carried out on ice. Cells were then centrifuged at 1500 rpm for 5 minutes and resuspended in 1ml cold phosphate-buffered saline (PBS). Suspended cells were lysed by adding 10µl of TCA (trichloroacetic acid) and were vortexed for 1 min. The lysate stayed on ice for 30 min and then the protein was separated by centrifugation at 13200 rpm for 10 min in an Eppendorf microcentrifuge. The supernatant, containing the dNTPs, was removed and neutralized by adding 200µl Freon/TNO (Tri-n-octylamin) (7.8T Freon + 2.2T TNO). Aliquots of 100µl were periodated by adding 30µl of 4M methylamine solution (pH 7.5) 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 0.32mol/l 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.

3.2.7 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 ($P<0.05$). The calculations of dose response curves and combination effects were performed using the Calcusyn® software designed by Chou and Talalay (Biosoft, Ferguson, MO). The analytical method describes the interaction among drugs in a given combination. A combination index (CI) of 0.9-1.1 indicates additive affects of two inhibitors, a CI of <0.9 shows synergism and a CI of >1.1 signifies antagonism.

4 Results

4.1 Ellagic acid (EA)

4.1.1 Effect of EA on the growth of HL-60 cells

HL-60 cells were seeded in T-25 cm^2 tissue culture flasks and were incubated with increasing concentrations of 10, 20, 30, 40 and 50μM at 37°C. After 24, 48 and 72h, cell counts were determined using the microcellcounter CC-110.
EA inhibited the growth of HL-60 cells with an IC_{50} value (IC_{50} = 50% growth inhibition of tumor cells) of 35μM after 72h (Figure 7).

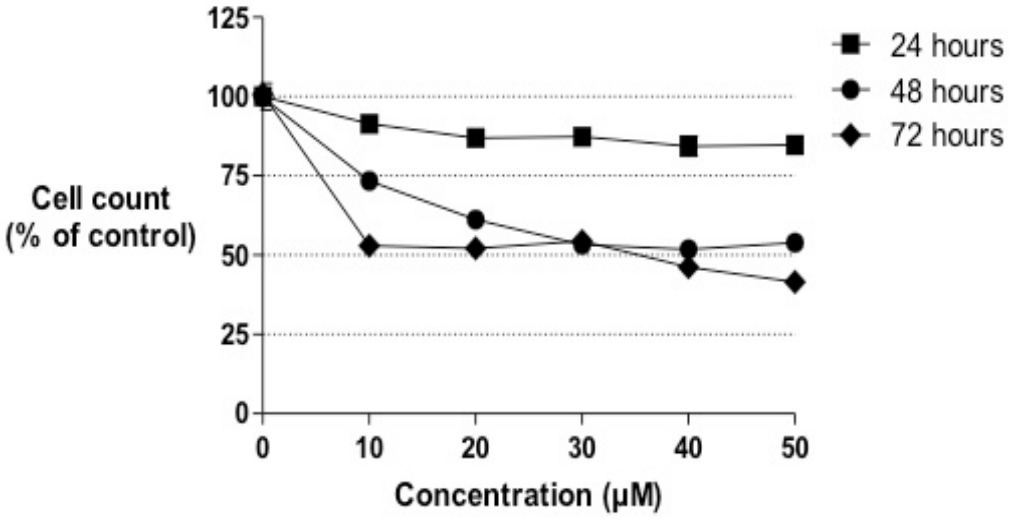

Figure 7: Growth inhibition of HL-60 cells after incubation with EA for 24, 48 and 72h

Effect of EA on the growth of HL-60 cells alone and in combination with Ara-C

To investigate the combination effect of EA with Ara-C, HL-60 cells were seeded at a concentration of 0.15 x 10^6 per ml and were simultaneously or sequentially incubated with various concentrations of EA (10, 20 and 30μM) and Ara-C. When treated simultaneously, two out of nine combinations caused synergism whereas the other seven combinations resulted in an additive effect (Table 1). Sequential application of EA and Ara-C generated synergistic effects in four out of nine drug combinations; four other combinations yielded an additive effect, whereas one combination resulted in slight antagonism (Table 2).

Table 1: Combination effects of EA and Ara-C in HL-60 cells employing a simultaneous growth inhibition assay

Compound	Concentration (µM/nM)	Cell number (% of control)	Predicted value*	Combination index**
EA (A)	10	86.6		
in µM	20	57.0		
	30	48.3		
Ara-C (B)	5	79.1		
in nM	10	61.1		
	20	44.6		
EA + Ara-C	10 5	63.2	68.5	1.015
EA + Ara-C	10 10	49.2	52.9	0.977
EA + Ara-C	10 20	37.8	38.6	1.088
EA + Ara-C	20 5	42.5	45.1	0.875***
EA + Ara-C	20 10	38.9	34.8	1.006
EA + Ara-C	20 20	28.2	25.4	0.992
EA + Ara-C	30 5	28.6	38.2	0.819***
EA + Ara-C	30 10	27.3	29.5	0.919
EA + Ara-C	30 20	23.5	21.6	1.025

Cells were simultaneously treated with EA and Ara-C for 72h. Afterwards, the cell count was determined.
* Predicted Value: (%A x %B) / 100
** Combination indices according to the equation of Chou and Talalay
***Synergistic combination effect

Table 2: Combination effects of EA and Ara-C in HL-60 cells employing a sequential growth inhibition assay

Compound	Concentration (μM/nM)	Cell number (% of control)	Predicted value*	Combination index**
EA (A)	10	96.5		
in μM	20	94.2		
	30	87.7		
Ara-C (B)	5	86.9		
in nM	10	83.6		
	20	76.3		
EA + Ara-C	10 5	87.3	83.8	1.285
EA + Ara-C	10 10	81.7	80.7	1.079
EA + Ara-C	10 20	74.4	73.6	0.916
EA + Ara-C	20 5	84.0	81.9	1.055
EA + Ara-C	20 10	78.5	78.8	0.939
EA + Ara-C	20 20	71.0	71.8	0.797***
EA + Ara-C	30 5	78.0	76.2	0.777***
EA + Ara-C	30 10	69.5	73.3	0.580***
EA + Ara-C	30 20	63.4	66.9	0.555***

Cells were sequentially incubated with EA for 24h and with Ara-C for 48h. Subsequently, the cell count was measured.
* Predicted Value: (%A x %B) / 100
** Combination indices according to the equation of Chou and Talalay
***Synergistic combination effect

4.1.2 Cell cycle distribution analysis after treatment with EA

HL-60 cells were incubated and treated with 60, 90 and 120μM of EA for 24h and with 30, 45 and 60μM of EA for 48h. Treatment of HL-60 cells with 60μM EA for 24h caused an accumulation in S-phase, thereby increasing the cell population from 22.3% to 38.8%. EA concentrations of 90 and 120μM led to a cell population in the S-phase of 32.1% and 29.5%, respectively. G0-G1 phase cells decreased from 63.0% to 43.9% when treated with 60μM EA (Figure 8).

When treated with increasing concentrations of EA for 48h, no significant changes in the cell cycle distribution could be observed when compared to untreated control cells (Figure 9).

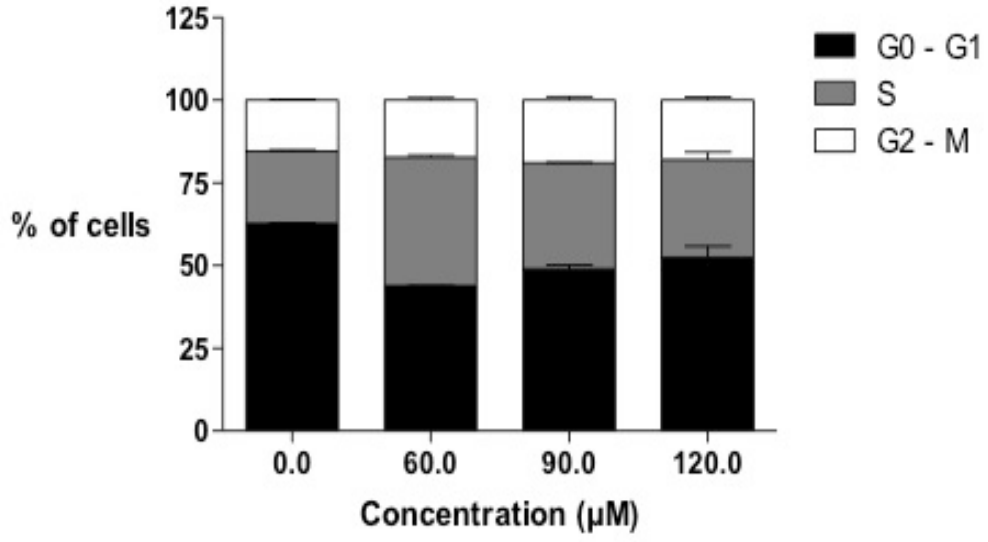

Figure 8: Cell cycle distribution in HL-60 cells after incubation with EA for 24h

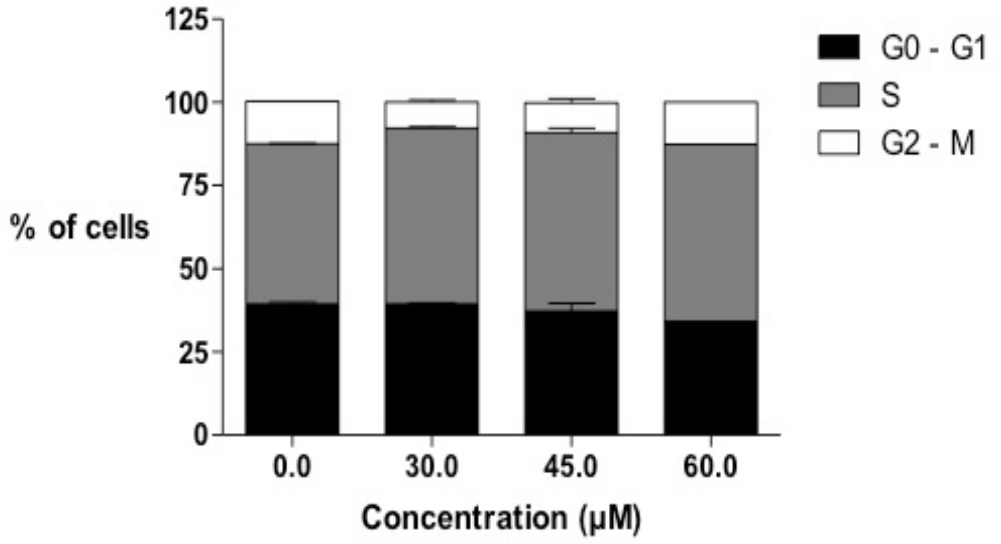

Figure 9: Cell cycle distribution in HL-60 cells after incubation with EA for 48h

4.1.3 Antioxidant activity of EA

The free radical-scavenging activity of EA was determined using the DPPH•-assay. After incubation with EA for 15 min, the inhibition of 50% of DPPH activity was observed at 6.4µM (Table 3). Ascorbic acid and α-Tocopherol served as reference compounds, with IC_{50} values of 21.5µM and 16.4µM, respectively.

Table 3: Radical scavenging activity of EA after incubation for 15 min, given as IC_{50} value

Compound	**IC_{50} (µM)**
EA	6.4
Ascorbic acid	21.5
α-Tocopherol	16.4

4.1.4 Induction of apoptosis in HL-60 cells after treatment with EA

HL-60 cells were exposed to 60, 90 and 120μM EA for 24h and to 30, 45 and 60μM EA for 48h. Cells were double stained with Hoechst 33258 and propidium iodide in order to analyze apoptosis induction. There occurred nearly no apoptotic events after incubation with EA for 24 and 48h. After 24h, only 6.8% of cells showed apoptosis when treated with 120μM of EA. When treated with EA for 48h, no noticeable induction of apoptosis could be observed (1.0% apoptosis in untreated control, 2.3% of apoptosis when treated with 60μM of EA).

4.1.5 Inhibition of incorporation of ^{14}C-labeled cytidine into DNA of HL-60 cells after treatment with EA

HL-60 cells were incubated with 60μM of EA for 48h in order to measure the RR *in situ* activity. Exposure to 60μM EA led to a decrease of 63.2% of ^{14}C-incorporation into nascent DNA after 48h, demonstrating a potential inhibition of the *in situ* activity of RR (Figure 10).

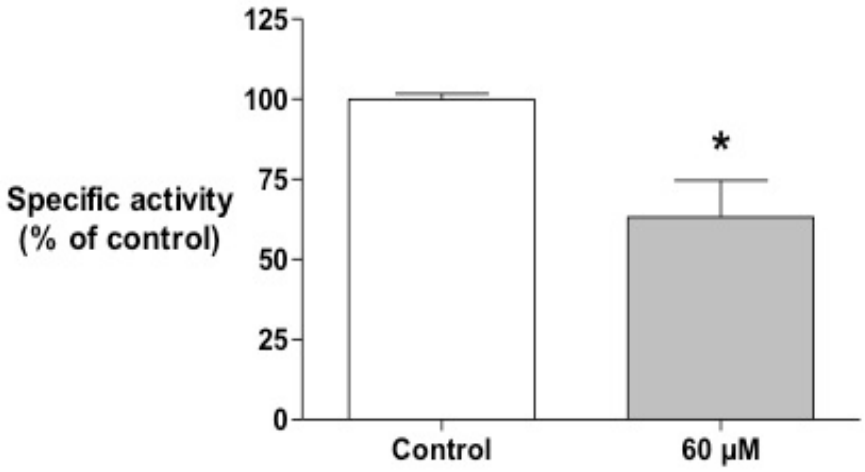

Figure 10: *In situ* measurement of ribonucleotide reductase activity in HL-60 cells after treatment with EA

4.1.6 Alterations in dNTP pools after treatment with EA for 24h

HL-60 cells were treated with 60, 90, and 120μM EA for 24h. In order to investigate dNTP pool sizes, a HPLC method as described in the material and methods section was used. Normal RR activity ensures balanced dNTP pools, whereas an inhibition of that enzyme can lead to pool size alterations and imbalances.

Treatment of HL-60 cells with EA caused shifts of intracellular dNTPs at all three concentrations. Incubation with 60μM EA resulted in a significant decrease of intracellular dCTP to 48.9% when compared to untreated controls. This observation is in line with the *in situ* activity of RR when HL-60 cells were treated with 60μM EA. Treatment with 90μM EA resulted in significant depletions of dCTP and dTTP concentrations to 71.2% and 79.7%, respectively. Incubating cells with 120μM EA decreased dATP pool sizes to 50.2% of control values. dCTP and dTTP pools remained unaltered. All dGTP pools remained outside the detection capability of the method (Figure 11).

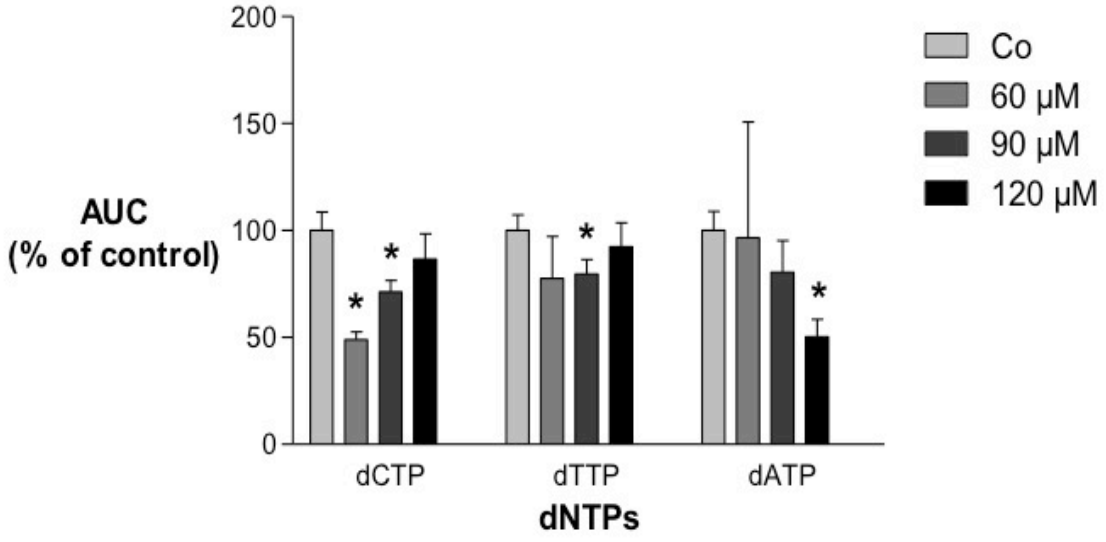

Figure 11: Concentration of dNTP pools in HL-60 cells after treatment with EA for 24h

4.2 Epigallocatechin gallate (EGCG)

4.2.1 Effect of EGCG on the growth of HL-60 cells

HL-60 cells were seeded in T-25 cm^2 tissue culture flasks and were incubated with 10, 20, 30, 40 and 50μM EGCG at 37°C. After 24, 48 and 72h, cell counts were determined using the microcellcounter CC-110.
EGCG inhibited the growth of HL-60 cells with IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) of 26μM after 48 and 72h, respectively (Figure 12).

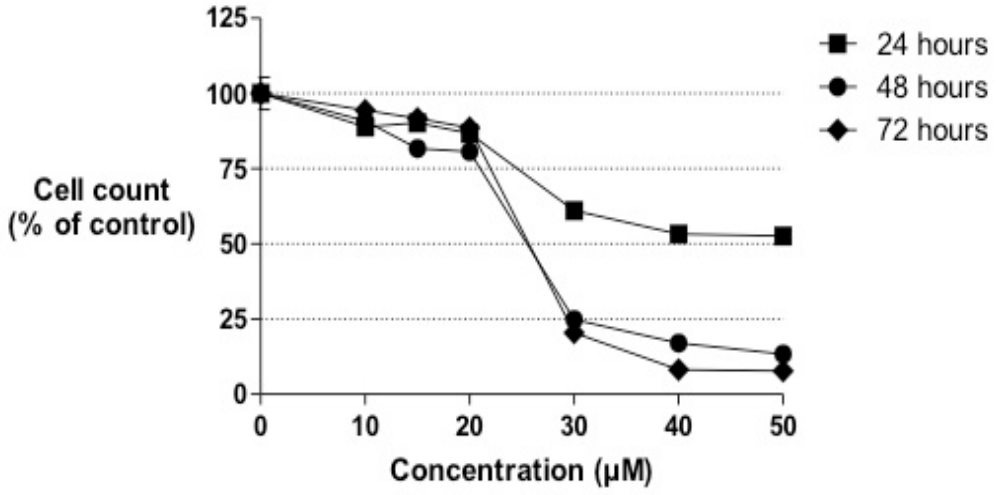

Figure 12: Growth inhibition of HL-60 cells after incubation with EGCG for 24, 48 and 72h

Effect of EGCG on the growth of HL-60 cells alone and in combination with Ara-C

To investigate the combination effect of EGCG with Ara-C, HL-60 cells were seeded at a concentration of 0.15 x 10^6 per ml and were sequentially incubated with various concentrations of EGCG (15, 20 and 25μM) and Ara-C. None of the nine observed drug combinations generated synergistic effects, whereas six out of nine combinations yielded additive effects. Three drug combinations showed antagonistic behavior (Table 4).

Table 4: Combination effects of EGCG and Ara-C in HL-60 cells employing a sequential growth inhibition assay

Compound	Concentration (μM/nM)	Cell number (% of control)	Predicted value*	Combination index**
EGCG in μM	15	93.2		
	20	75.6		
	25	23.1		
Ara-C (B) in nM	5	87.3		
	10	83.0		
	20	71.6		
EGCG + Ara-C	15 5	81.3	81.3	1.328
EGCG + Ara-C	15 10	73.6	77.4	1.853
EGCG + Ara-C	15 20	58.7	66.8	1.136
EGCG + Ara-C	20 5	55.9	66.0	1.030
EGCG + Ara-C	20 10	53.0	62.8	1.078
EGCG + Ara-C	20 20	39.7	54.2	1.004
EGCG + Ara-C	25 5	15.8	20.2	0.914
EGCG + Ara-C	25 10	17.8	19.2	0.941
EGCG + Ara-C	25 20	14.4	16.6	0.916

Cells were sequentially incubated with EGCG for 24h and with Ara-C for 48h. Afterwards, the cell count was determined.
* Predicted Value: (%A x %B) / 100
** Combination indices according to the equation of Chou and Talalay
***Synergistic combination effect

4.2.2 Cell cycle distribution analysis after treatment with EGCG

HL-60 cells were incubated and treated with 10, 20, 30, 40 and 50μM of EGCG for 24h. Treatment with 50μM EGCG caused an accumulation of HL-60 cells in G0-G1 phase, elevating the cell population of 48.2% when compared to untreated controls (34.6%). Simultaneously, a depletion of cells in the S-Phase from 48.5% to 40.1% and from 16.9% to 11.7% in G2-M phase could be observed (Figure 13).

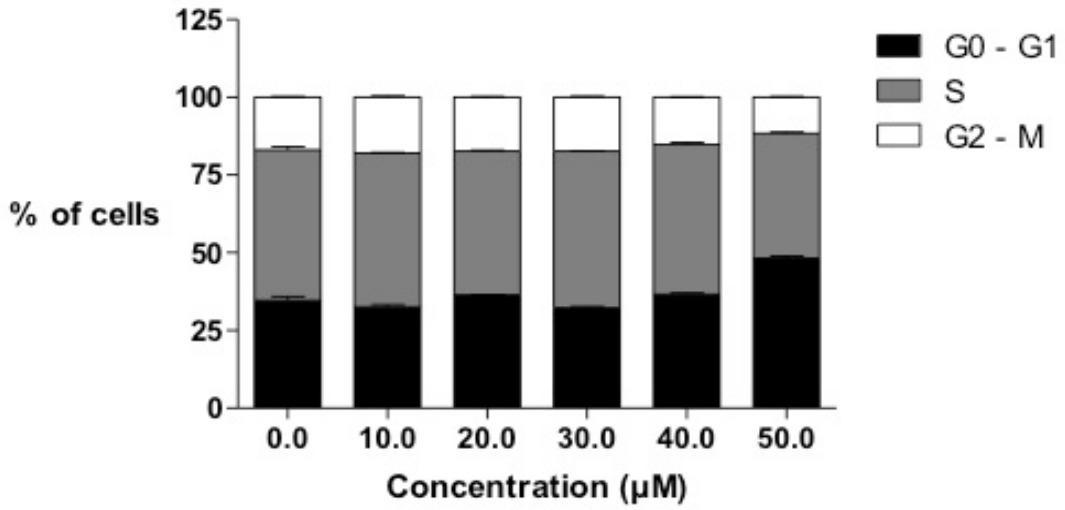

Figure 13: Cell cycle distribution in HL- 60 cells after incubation with EGCG for 24h

4.2.3 Antioxidant activity of EGCG

The free radical-scavenging activity of EGCG was experimentally determined utilizing a DPPH•-assay. The inhibition of 50% of DPPH• activity was given as IC_{50} value. After incubation for 15 min, EGCG inhibited DPPH• activity with an IC_{50} value of 5.7μM (Table 5). Ascorbic acid and α-Tocopherol were used as reference compounds, showing IC_{50} values of 21.5μM and 16.4μM, respectively.

Table 5: Radical scavenging activity of EGCG after incubation for 15 min, given as IC_{50} value

Compound	**IC_{50} (μM)**
EGCG	5.7
Ascorbic acid	21.5
α-Tocopherol	16.4

4.2.4 Induction of apoptosis in HL-60 cells after treatment with EGCG

HL-60 cells were exposed to 10, 20, 30, 40 and 50μM EGCG for 24 and 48h. Cells were double stained with Hoechst 33258 and propidium iodide to analyze if apoptotic cell death was induced. After 24 and 48h, HL-60 cells showed early and late apoptotic stages when treated with EGCG. Treatment of cells with 40μM EGCG resulted in 70% apoptotic cells and 50μM EGCG even caused 83.6% of cells to undergo early and late apoptosis. Also after 48h, 56.3% and 84.3% of HL-60 showed apoptosis upon treatment with 40μM and 50μM EGCG, respectively (Figures 14 and 15). Fluorescence microscopy investigations illustrate HL-60 cells at different stages of apoptosis when treated with EGCG (Figure 16).

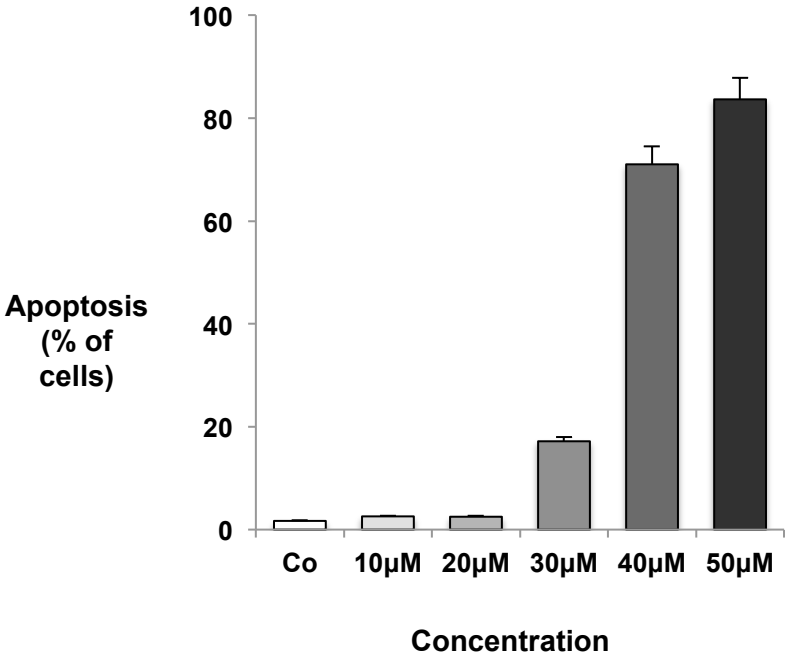

Figure 14: Induction of apoptosis in HL-60 cells after incubation with EGCG for 24h

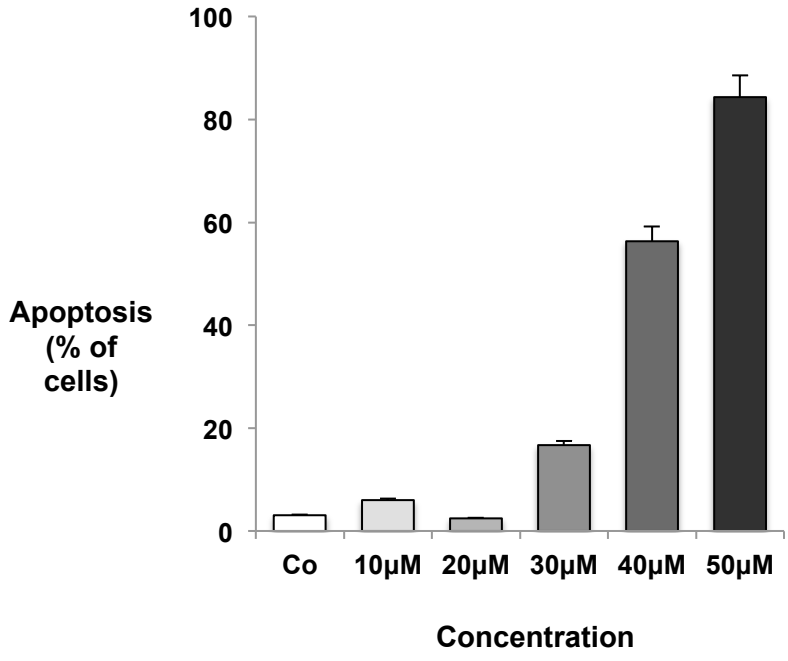

Figure 15: Induction of apoptosis in HL-60 cells after incubation with EGCG for 48h

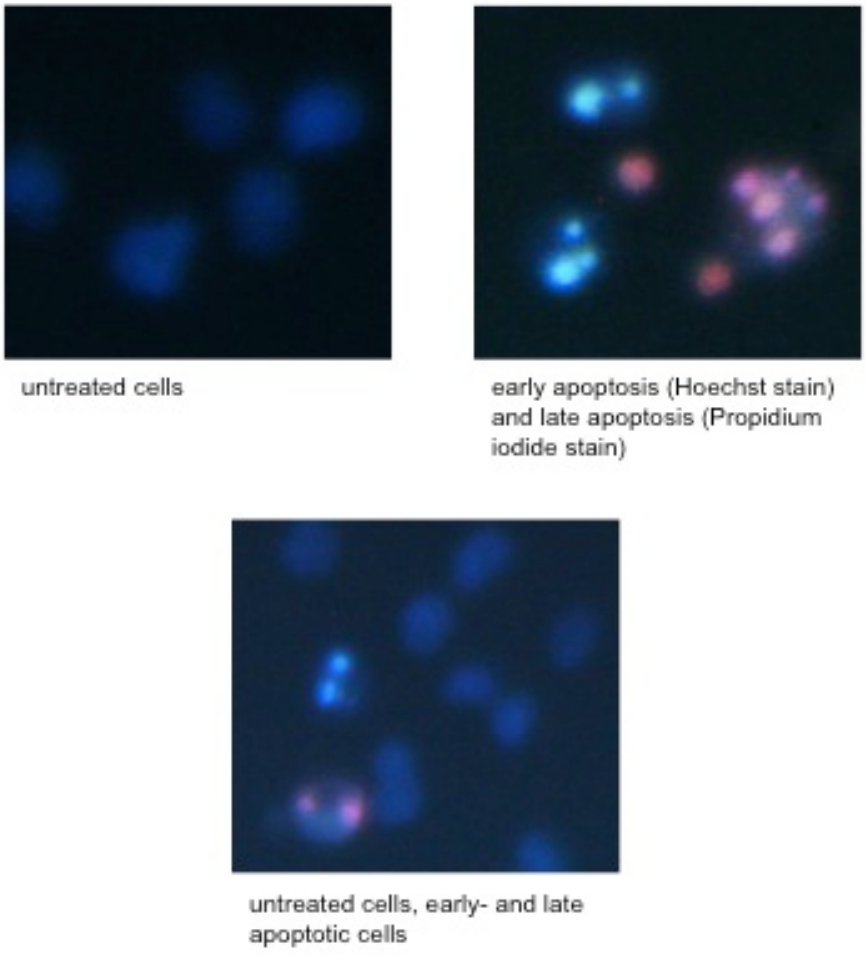

Figure 16: Cellular morphology of HL-60 cells treated with EGCG for 24 and 48h. Cells were double stained with Hoechst 33258 and propidium iodide

4.2.5 Inhibition of incorporation of ^{14}C-labeled cytidine into DNA of HL-60 cells after treatment with EGCG

To measure the activity of RR *in situ*, HL-60 cells were incubated with 20μM EGCG for 24h. EGCG treatment led to a decrease of DNA-associated radioactivity to 45.6% when compared to control (Figure 17). This indicates a reduced cytidine metabolism and hence an inhibition of RR *in situ* activity. As natural antioxidative substances like EGCG are known for their ability to scavenge radicals, inhibition of RR might appear due to the destruction of the essential tyrosyl radical being harbored by subunit R2.

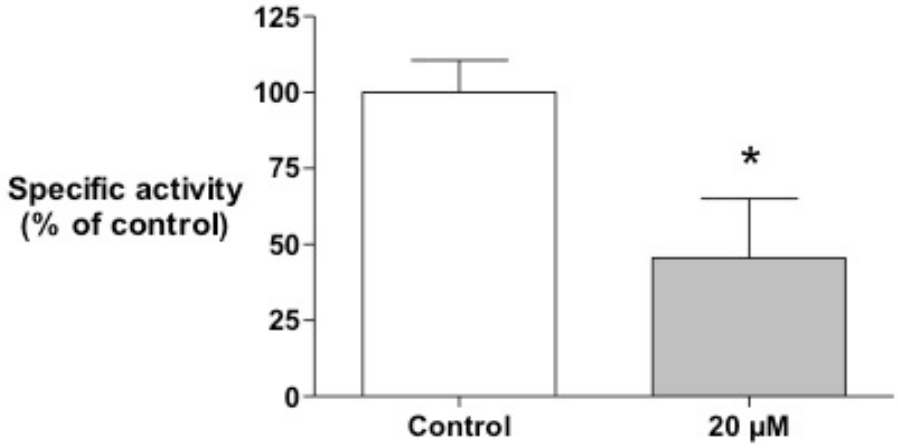

Figure 17: *In situ* measurement of ribonucleotide reductase activity in HL-60 cells after treatment with EGCG

4.2.6 Alterations in dNTP pools after treatment with EGCG for 24h

Incubation of HL-60 leukemia cells with 40μM EGCG led to significant changes of intracellular dCTP and dATP concentrations. dCTP pools increased up to 141.4% of control values, whereas a significant depletion of dATP pool sizes of 37.1% was detected. All dGTP pools remained beyond detectability limit of the method (Figure 18). These findings correlate with the above-mentioned inhibition of RR *in situ* activity and hence perturbations of dNTP metabolism.

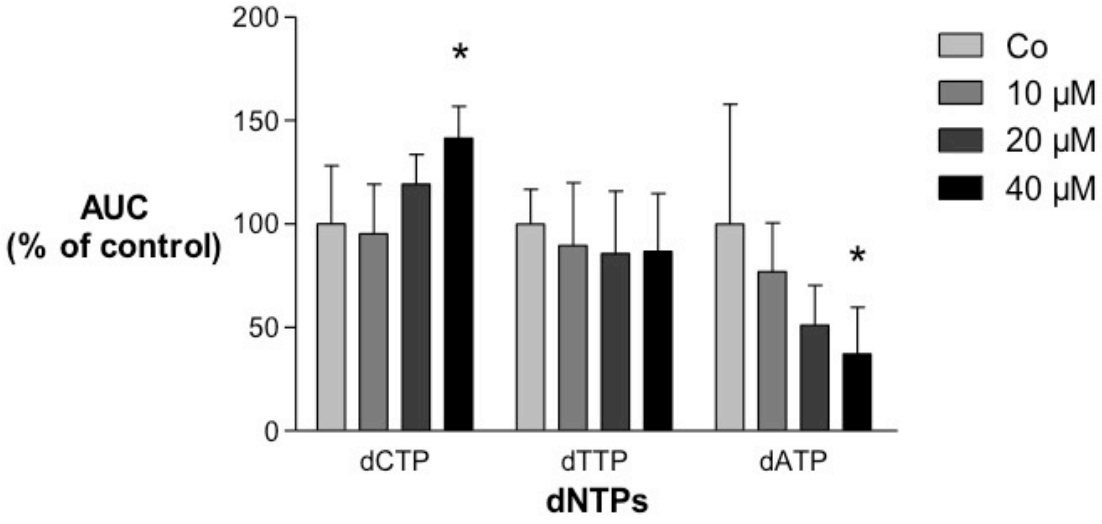

Figure 18: Concentration of dNTP pools in HL-60 cells after treatment with EGCG for 24h

5 Discussion

EA

To investigate the effect of EA on the human HL-60 promyelocytic leukemia cell line, a number of experiments have been conducted.

In normal cells, RR provides a balanced supply of dNTPs used for DNA synthesis in a regulated manner. As cancer cells need large amounts of these building blocks due to their augmented proliferation potential, RR activity is supposed to be highly upregulated. Thus, inhibition of the enzyme constitutes a suitable mechanism to prevent malignant cells to rapidly overgrow (Saiko et al., 2008).

Growth inhibition of HL-60 cells was observed after 72h with EA showing an IC_{50} value of 35 μM, demonstrating its cytotoxic activity.
Further, additive effects and even synergism, leading to intensified HL-60 cell growth inhibition, could be detected when higher concentrations of EA and the cytostatic agent Ara-C were administered simultaneously as well as sequentially. Existing data also revealed that EA applied in combination with other compounds yielded synergistic effects (Mertens-Talcott & Percival, 2005). These findings indicate that combinations of polyphenolic compounds together with already existing drugs could enhance antineoplastic effects in tumor cells.

Cell cycle distribution results displayed that HL-60 cells are accumulating in S-phase after 24h. After 48h, no significant changes in the cell cycle could be observed, suggesting that there might exist a reversible mechanism that leads to different phase arrests throughout the cell cycle in HL-60 cells when treated with EA. Similar observations have been reported in former studies as well, where cell cycle kinetics in human leukemia cells normalized after 48h of treatment (Mertens-Talcott & Percival, 2005). Furthermore, no apoptotic events were detected in cancer cells after treatment.

S-phase accumulation of cancer cells after 24h followed by normalized cell cycle

kinetics after 48h leads to the assumption that some cells may recover from treatment after a period of time. Cell cycle arrest is supposed to protect cells to undergo apoptosis. As there occurred no apoptotic events in HL-60 cells when treated with EA, the transient arrest could have been responsible for their protection against apoptosis. EA is therefore assumed to cause temporary cell cycle arrest rather than cell death.

Due to its chemical scaffold, EA is presumed to possess antioxidative properties. The OH groups are able to donate hydrogen atoms or electrons that consequently can lead to neutralization of ROS (Priyadarsini et al., 2002). The performed DPPH assay showed that EA could scavenge free radicals with an IC_{50} value of 6.4μM, expecting it to act as a competent antioxidant compound. The reference compounds ascorbic acid and α-tocopherol, known for their role as antioxidants, showed IC_{50} values of 21.5μM and 16.4μM, respectively.

Moreover, 60μM EA caused an inhibition of RR activity *in situ*. Further, dNTP pool alterations could be observed when HL-60 leukemia cells were treated with different concentrations of EA. In addition to these observations, the potential antioxidative capability of EA supports the supposition of EA being able to scavenge the free radical in subunit R2 of RR, thus leading to an interruption of the enzymes activity. All these results estimate EA to function as an inhibitor of the *in situ* activity of RR.

The findings are also in line with cell cycle distribution analysis that led to an S-phase arrest of HL-60 cells after 24h. As RR is the rate-limiting enzyme for S-phase transition, its inhibition might be responsible for an imbalance in *de novo* DNA synthesis and hence the accumulation of cells in S-phase.

According to these results, EA represents a promising antioxidant compound found within a wide range of nourishments. Hence, further *in vitro* and *in vivo* studies on EA will shed light on its role as a chemopreventive agent.

EGCG

Being the most abundant polyphenol in green tea, EGCG showed dose-dependent cytotoxicity in HL-60 cells leading to growth inhibition with an IC_{50} of 26μM after 48 and 72h.

Although no synergistic effects were detected when EGCG was combined with Ara-C, some combinations inhibited cell growth additively. Huo et al. reported that EGCG is supposed to have poor bioavailability. As additive effects could be observed when higher dosed combinations of EGCG together with Ara-C were administered, these findings lead to the assumption that higher concentrations of EGCG could result in enhanced inhibition of tumor cell proliferation due to better cellular uptake.

After incubation with EGCG for 24h, cells accumulated in G0-G1 phase when treated with 50μM EGCG. Besides, a depletion of cells in S-phase was detected. Furthermore, there occurred early and late apoptotic events in HL-60 cells that were incubated with 40 and 50μM EGCG for 24 and 48h, respectively. Over 80% of cells underwent apoptosis. Similar to these findings, other studies also revealed a G0-G1 phase arrest of leukemic cells being followed by apoptosis when treated with EGCG. Moreover, this research group demonstrated that a breakdown of mitochondrial membrane potential could be responsible for the induction of apoptosis due to the release of cyctochrome *c* and subsequent activation of caspases. Elevated levels of intracellular ROS are as well supposed to occur at apoptotic events (Nakazato et al., 2005). It is therefore believed that EGCG possibly undergoes intracellular oxidation that could eventuate in the formation of ROS that hence causes apoptotic cell death. These results would underlie the pro-oxidant activity ascribed to EGCG.

Oxidative stress along with the presence of ROS may result in DNA damage and dysfunctional cell metabolism (Kang et al., 2011). Additional to above-mentioned outcomes, EGCG could demonstrate its potential role as antioxidant. The radical scavenging activity was examined employing a DPPH assay and resulted in an IC_{50} value of EGCG of 5.7μM when compared to 21.5μM and 16.4μM of ascorbic

acid and α-tocopherol reference antioxidants, respectively. Moreover, EGCG inhibited RR *in situ* activity at a concentration of 20μM after 24h. In line with these findings, significant dNTP pool size imbalances with highly increased dCTP concentrations as well as strongly depleted dATP levels were detected. That inhibition of RR activity might be due to the ascribed antioxidant capability of EGCG leading to a destruction of the essential tyrosyl radical of subunit R2.

These aforementioned results conclude that EGCG might also have an impact on the diiron center of R2 as it acts as a metal chelator, thereby inducing enzyme instability. Emphasizing an interaction between subunit R2 and EGCG, the diiron center could also contribute to observed pro-oxidative effects of EGCG, as transition metal reduction is supposed to be linked to phenolic oxidation (Lambert & Elias, 2010).

Taken together, growth arrest of HL-60 cells, cell cycle perturbations followed by apoptosis as well as a repression of RR activity marked by alterations in dNTP pools highlight the importance of that essential green tea component in contributing to malignant cancer cell death, hence underscoring its role as a promising compound that could be used in cancer prevention.

Both, antioxidant and pro-oxidant roles of EGCG need in depth investigations to unravel the precise molecular mechanisms of that polyphenolic substance. In addition, synthetic analogs of EGCG with better bioavailability and longevity together with the possibility of applying higher concentrations are supposed to yield in more efficient chemopreventive active agents.
On that account, *in vivo* studies as well as preclinical investigations will provide important information on biological activities of EGCG that might qualify it as an effective candidate for cancer therapy.

6 Abstract

The urgent need for new cancer therapies and preventive agents against the outbreak of malignant diseases is currently displayed by intensive research in this field.
Ribonucleotide reductase (RR) is an enzyme responsible for the conversation of ribonucleotides into deoxyribonucleotides. Hence, it possesses an outstandingly important role in the *de novo* DNA synthesis that occurs during S-phase of the cell cycle. Overexpression of RR in malignant cancer cells makes it a suitable target for chemotherapeutic treatments.

In this master thesis, two polyphenolic compounds were studied on their capability of acting as anticancer substances. Ellagic acid (EA) is a polyphenolic acid and occurs in a wide range of natural foods like berries, grapes and nuts. Epigallocatechin gallate (EGCG) belongs to the group of flavanols and is the most widespread component in green tea.
Their biological effects were investigated in human HL-60 promyelocytic leukemia cell line. Both natural occurring polyphenols were supposed to exert auspicious properties as chemopreventive agents.

In fact, both compounds were discovered to constitute potent inhibitors of RR activity, which was reflected by a decreased incorporation of ^{14}C-labeled cytidine into nascent DNA of tumor cells. In addition, HL-60 leukemia cells revealed alterations in dNTP pool sizes and both substances also hold radical scavenging potential.

EA caused cancer cells to transiently accumulate in S-phase of the cell cycle but proximately were able to recover, leading to the assumption that EA acts as an inhibitor of cell cycle progression rather than as an inducer of cell death. Investigations on EGCG illustrated that leukemic cells were arrested in G0-G1 phase and most of them subsequently underwent apoptosis.

Further, EA in combination with a well established first-line chemotherapeutic agent Cytarabine (Ara-C) exhibited a synergistic antineoplastic impact, whereas that effect could not be observed when EGCG was combined with Ara-C.

Due to these findings, EA and particularly EGCG portray potential candidates for ongoing and comprehensive research studies.

7 Zusammenfassung

Der fortwährende Bedarf an neuen Krebstherapien wie auch der Einsatz präventiver Arzneimitteln zur Unterbindung von Krankheitsausbrüchen führt gegenwärtig zu intensiver Forschung auf diesem Sachgebiet.
Die Ribonukleotidreduktase (RR) ist ein Enzym welches für die Umwandlung von Ribonukleotiden in Desoxyribonukleotide verantwortlich ist. Auf Grund dieser Eigenschaft nimmt es eine überaus wichtige Position in der *de novo* DNA Synthese ein, welche während der S-Phase des Zellzyklus stattfindet. Da die RR in malignen Krebszellen häufig überexprimiert ist, stellt sie ein sehr geeignetes Angriffsziel für chemotherapeutische Behandlungen dar.

Im Zuge dieser Masterarbeit wurden zwei polyphenolische Verbindungen heran gezogen, um sie auf ihre Fähigkeit als antikanzerogene Substanzen zu untersuchen. Ellagsäure (ES) ist eine Polyphenolsäure, die in vielerlei Nahrungsmitteln, insbesondere in Obst- und Gemüsesorten wie Beeren, Weintrauben und Nüssen vorkommt. Epigallocatechingallat (EGCG) gehört zur Gruppe der Flavanole und stellt die bedeutendste Komponente des Grüntees dar. Die biologischen Effekte dieser Naturstoffe wurden in humanen HL-60 Promyelozytenleukämiezellen erforscht. Beide natürlich vorkommenden Polyphenole gelten als vielversprechende Stoffe für die Krebsforschung, da ihnen auch eine chemopräventive Wirkung zugewiesen wird.

Tatsächlich bewirkten beide Substanzen eine Hemmung der Aktivität der RR, was mit einem verminderten Einbau von ^{14}C-markierten Cytidin in die DNA der Tumorzellen einherging. Des Weiteren kam es in HL-60 Zellen zu deutlichen Veränderungen hinsichtlich der Konzentrationen der dNTP Pools. Auch konnte nachgewiesen werden, dass beide Verbindungen über eine starke antioxidative und somit radikalfangende Wirkung verfügten.

ES verursachte eine vorübergehende Akkumulation von Krebszellen in der S-Phase des Zellzyklus, welche jedoch im Stande waren, sich nach geraumer Zeit wieder zu erholen. Dies legt den Schluss nahe, dass ES eher als Inhibitor des

Zellzyklus fungiert und nicht als Auslöser des Zelltodes anzusehen ist. Untersuchungen mit EGCG ergaben hingegen, dass Leukämiezellen in der G0-G1 Phase arretiert wurden und anschließend in Apoptose gingen.

Ferner ging aus den durchgeführten Experimenten hervor, dass ES in Kombination mit Cytarabin (Ara-C), welches als First-Line Zytostatikum breite Anwendung findet, einen synergistischen antineoplastischen Effekt erzielen konnte. Diese Wirkung konnte jedoch bei gleichzeitiger Gabe von EGCG und Ara-C nicht beobachtet werden.

Hinsichtlich dieser Resultate repräsentieren ES und EGCG potentielle Kandidaten für weiter gehende und umfassendere Forschungsstudien.

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