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A key antitumor mechanism in human cancer cells”

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## ABBREVIATION INDEX

5-FU	5-fluorouracil
ABNM-13	N-hydroxy-2-(anthracene-2-yl-methylene)-hydrazinecarboximidamide
Akt	serine/threonine-specific protein kinase
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
Apaf-1	apoptotic protease activating factor 1
APL	acute promyelocytic leukemia
Ara-C	cytarabine
AsPC-1	pancreatic cancer cell line
ATCC	American type culture collection
ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia mutated and Rad3 related
Bax	co-factor of the tumor suppressor protein p53
Bcl-2	B-cell lymphoma 2
BRCA1/BRCA2	tumor suppressor gene; breast cancer 1 and 2
BxPC-3	pancreatic cancer cell line
Cdc2	cell division control protein 2
Cdc25A/B/C	cell division control 25 A/B/C
CDK	cyclin-dependent kinases
Chk1/Chk2	checkpoint kinases 1 and 2
Cip/Kip	class of cyclin-dependent kinases inhibitors
CKI	cyclin-dependent kinases inhibitors
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CRT	chemoradiotherapy
dATP	deoxyadenosine triphosphate
dCK	deoxycytidine kinase
dFdC	gemcitabine
dGTP	deoxyguanosine triphosphate
DIG	digalloyl-resveratrol
DNA	deoxyribonucleotide acid
dNTP	deoxyribonucleotide triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl (free radical)
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
FC	fludarabine plus cyclophosphamide
FDR	fixed-dose rate regimen
FdUMP	fluorodeoxyuridine monophosphate
G <sub>0</sub> , G <sub>1</sub> , G <sub>2</sub>	gap phases of the cell cycle
GA	gallic acid (3,4,5-trihydroxybenzoic acid)
H <sub>3</sub> PO <sub>4</sub>	ortho-phosphoric acid
HER2/neu	human epidermal growth factor receptor 2
HL-60	acute promyelocytic leukemia cell line
HO	Hoechst Dye 33258
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase

HT-29	colon cancer cell line
HTLV-1	human T-lymphotropic virus type I
IC <sup>50</sup>	concentration causing 50% inhibition
INK4	class of cyclin-dependent kinases inhibitors
KITC	<i>N</i> -hydroxy- <i>N'</i> -(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxy-benzamidine
M	molar
MAPK	mitogen-activated protein kinase
MeOH	methanol
mM	millimolar
M-phase	mitosis
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
p16	member of INK4 family
p21 <sup>cip</sup>	CDK inhibitor 1
P38MAPK	p38 mitogen-activated protein kinase
p53	tumor suppressor protein/transcription factor
P53R2	R2 homolog
PAGE	polyacrylamide gel electrophoresis
PANC-1	pancreatic cancer cell line
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline/tween
pH	pondus hydrogenii
PI	propidium iodide
PIC	protease Inhibitor Cocktail
PML	promyeloid leukemia
PMSF	phenylmethylsulfonyl fluoride
PVDF	membranes for Western Transfer and Sequencing
QSAR	quantitative structure-activity relationship
R1	large $\alpha_2$ -homodimer of ribonucleotide reductase
R2	small $\beta_2$ -homodimer of ribonucleotide reductase
RAR- $\alpha$	retinoic acid receptor- $\alpha$
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	cell culture medium (Roswell park memorial institute)
RR	ribonucleotide reductase
RV	resveratrol (3,4,5-trihydroxystilbene)
SCT	stem-cell transplantation
SDS	sodium dodecyl sulphate
Ser	serin
S-phase	DNA-synthesis
TBS	tris buffered saline
TBS/T	tris buffered saline/tween



## 1. Introduction

Cancer is the major public health problem and remains the leading cause of death worldwide. The United States National Institute of Cancer (NIC) estimates approximately 1.5 million people diagnosed with cancer each year ([www.cancer.gov](http://www.cancer.gov)). Thus, there exists an urgent need for the discovery or development of novel compounds representing antineoplastic effects.

Despite the incredible effort invested in the development of novel cytostatic and regulatory agents, cytotoxic chemotherapy associated with relatively high toxicity remained the only option in the treatment of advanced/metastatic cancer over the past few decades. The quest for better, more efficacious and less toxic approaches to the delivery of cytotoxic agents has intensified. Today, about 60% of cytostatic agents in Western medicine are metabolites of naturally occurring compounds (Newman and Cragg, 2007). New methodologies have dramatically improved drug screening and synthesis of novel analogs with more favorable performance characteristics than their predecessors. Great progress was made in identifying and analyzing new anticancer agents with enhanced efficacy while at the same time exerting less toxicity. Many of these investigated drugs already showed a broad spectrum of beneficial physiological and antineoplastic effects, the latter through induction of apoptosis and inhibition of several enzymes of DNA synthesis in a multitude of cancer cell lines. In this diploma thesis, the following compounds were investigated for their anticancer activity in various human tumor cell lines:

ABNM-13 is a newly designed N-hydroxy-N'-aminoguanidine. The compound has been designed by a 3D molecular space modeling technique aiming at the inhibition of ribonucleotide reductase (RR) activity in human HL-60 promyelocytic leukemia cells. Treatment with various other compounds have already shown that diminished RR activity causes alterations of the dNTP pool balance (Szekeres et al, 1997) and decrease of incorporation of labeled cytidine into DNA (Szekeres et al, 1994).

Digalloyl-resveratrol (DIG) is a novel synthetic ester of the polyhydroxy phenolic substances resveratrol (3,4,5-trihydroxystilbene; RV) and gallic acid (3,4,5-trihydroxybenzoic acid; GA). Both compounds are naturally occurring agents, which are present in several plants and are known to own disease preventive properties in various cancer cell systems (Horvath et al, 2006; Madlener et al, 2007).

Recent investigations have already demonstrated a significant growth inhibiting and RR inhibiting effect of DIG in human HT-29 colon cancer cells (Bernhaus et al, 2009). The biochemical effects of DIG were investigated in three different human pancreatic cancer cell lines.

Both agents were compared for their cytotoxicity employing growth inhibition and clonogenic assays. The induction of apoptosis was quantified using a Hoechst/propidium iodide double staining method and cell cycle distribution effects were evaluated by FACS. The question of whether these drugs inhibit the *in situ* activity of RR and/or affect the steady state deoxynucleosidetriphosphate pools (dNTPs), which are the products of RR metabolism, is addressed by incorporation of radio-labeled cytidine into DNA of tumor cells and by employing a specific HPLC method, respectively. Additionally, the combination effects of these compounds were examined. Therefore, agents were applied simultaneously or sequentially with clinically well-established anticancer drugs like Cytarabine (Ara-C) or Gemcitabine (dFdC). Following western blot analysis displayed a deeper insight into the underlying mechanisms of apoptotic and growth inhibiting pathways.

All investigations provide new information about the biological activities of synthetically designed ABNM-13 and DIG, synthesized of the two natural occurring compounds resveratrol and gallic acid. Therefore, these agents might contribute to the clinical establishment of new combined therapies of human malignancies.

## 2. Literature Survey

### 2.1. Ribonucleotide Reductase

In 2010 the discovery of ribonucleotide reductase (RR) celebrated its 50<sup>th</sup> anniversary (Reichard, 2010). Since then it has been proven that disturbance in its allosteric regulation is associated with malignant transformation and tumor cell proliferation. RR was therefore considered an important target for anticancer agents (Smith and Karp, 2003; Nocentini, 1996).

#### 2.1.1. Function and Structure

Ribonucleotide reductase (RR) catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are the basic units for DNA synthesis and DNA repair (Kolberg et al, 2004). *De novo* dNTP synthesis is an exactly regulated reaction; thus, RR is the rate-limiting enzyme in that mechanism. Imbalances in dNTP levels or in the production of equimolar amounts cause cell death or genetic abnormalities (Reichard, 1988).

The enzymatic activity of RR proceeds by free radical chemistry; due to the way this radical is generated, RR is divided into three different but related main classes. Each class is based on a different metal cofactor for radical generation (Nordlund and Reichard, 2006).

Class I enzymes are mainly found in eukaryotic organisms and can be further divided into three subclasses. The human RR is a tetrameric holoenzyme ( $\alpha_2\beta_2$ ) and belongs to Class Ia (Stubbe et al, 2003). It consists of a large  $\alpha_2$ -homodimer, called R1 and of a small  $\beta_2$ -homodimer, called R2 (figure 1).

R2 harbors an oxygen-linked dinuclear Fe(III) center to create a tyrosyl radical that is essential for the transient formation of a thiyl radical in R1.

R1 is the reductase subunit and contains redox active disulfides that take part in the reduction of the substrate. Furthermore it harbors allosteric sites for effector binding and an active site for substrate binding (Reichard, 2010; Shao et al, 2006).

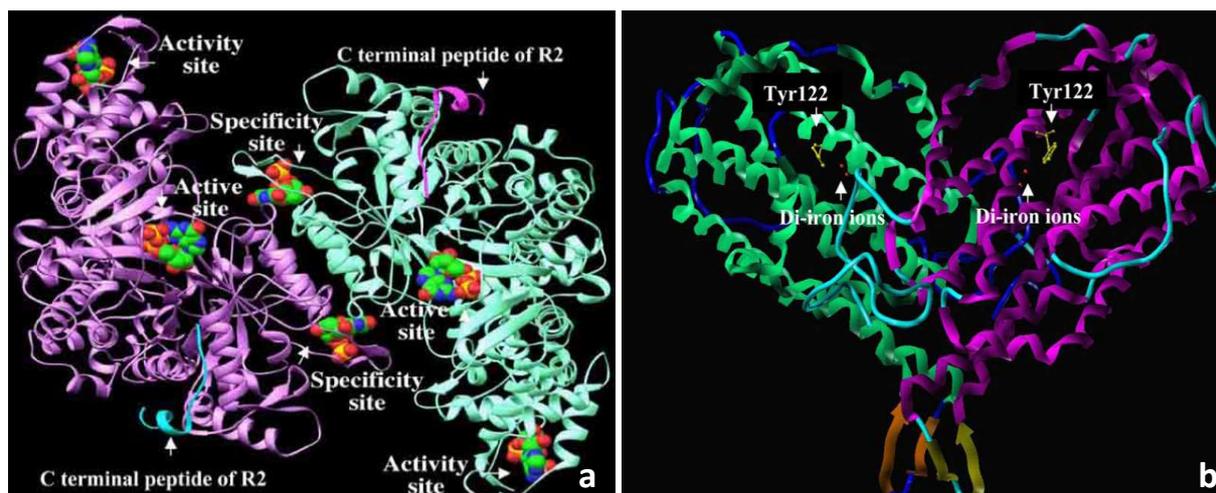


Figure 1: *E. coli* R1 protein dimer with C-terminal end of R2 (a). *E. coli* R2 protein dimer structure (b) (Shao et al, 2006).

### 2.1.2. Multiple Regulations

The activity of RR in mammalian cells can be regulated differently. To provide a balanced supply of dNTPs for DNA replication; to achieve this goal, the enzyme is regulated by a difficult allosteric regulation. The allosteric sites in the R1 subunit contain an activity site (A-site), a specificity site (S-site) and a hexamerization site (H-site).

ATP (activation) and dATP (feedback inhibition) bind to A-site for regulating overall enzyme activity. Binding of allosteric effectors (dATP, ATP, dTTP, dGTP) to S-site ensures correct proportion of dNTPs. ATP binding to the third bonding-site catalyzes the rearrangement of the active  $R1_6R2_6$  hexamer of RR (Shao et al, 2006).

Enzyme activity can also be regulated by R2 protein level. During the S-phase of the cell cycle, equal amounts of dNTPs are required. Therefore, the quantities of RR/transcription of the subunits in proliferating cells are closely linked with the cell cycle. While R1 shows constant expression level throughout the cell cycle, R2 accumulates in early S phase and afterwards rapidly degrades (Thelander et al, 1985).

### 2.1.3. A target for cancer chemotherapy

RR is responsible for the appropriate supply of the four dNTPs for DNA synthesis and is therefore an essential enzyme in the cell proliferation mechanism as already mentioned.

Inhibition of RR decreases dNTP amounts, *de novo* DNA synthesis and DNA repair and thus causes apoptosis and cell cycle arrest (Nocentini, 1996).

Cancer cells need great amounts of dNTPs for their increased cell proliferation. Hence, RR activity is increased and is in contrast associated with decreased adaptability and responsiveness. RR is therefore a more sensitive target for cytotoxic agents in tumor cells than in native cells (Nocentini, 1996).

Tanaka et al described a new RR gene, *p53R2* that is directly regulated by p53 transcription factor. p53 tumor suppressor protein regulates cell cycle by arresting at the G<sub>1</sub>/S regulation point or by initiating apoptosis. Expression of p53R2, a R2 homolog, is induced by  $\gamma$ -irradiation, UV or DNA damage (Tanaka et al, 2000).

Various investigations showed that two different pathways maintain the balanced supply of dNTPs: one way through the well-known R2 activity during the S/G<sub>2</sub> phase and the other way through p53R2. In that case, dNTPs are required for DNA repair in a p53-dependent manner (Yamaguchi et al, 2001; Lozano et al, 2000).

These findings increased the relevance of p53R2 in human cancer development. Various regulation mechanisms provide different targets enabling the development of subunit-specific agents for different applications and effects. For example, specific inhibition of p53R2 increases the sensitivity of tumor cells to cytotoxic agents (Lin et al, 2004).

#### **2.1.4. Human RR inhibitors**

For the last decades, various specific inhibitors of RR have been discovered. Some of them have found clinical applications as anti-tumor agents; others are still in clinical trials. RR inhibitors are distinguished between their chemical structure and properties and between their different targets and mechanisms of action. Inhibiting effects are caused by gene expression regulation or by protein inactivation.

Gemcitabine (dFdC) as well as Cytarabine (Ara-C) are substrate analogs that inactivate the enzyme by inhibition of R1 protein. Both inhibitors are first modified by deoxycytidine kinase (dCK) at the carbon 2' of ribose or arabinose and therefore recognized by RR as natural purine or pyrimidine nucleosides. Ara-C is modified to ara-CDP and ara-CTP while dFdC is further metabolized to dFdCDP and dFdCTP. Interaction with the active site of R1 leads to

deficient products that inactivate RR (van der Donk, 1998; Shao et al, 2006). The inhibition of RR is followed by a decrease of dNTPs and in further consequence by competition between normal dCTP and dFdCTP/ara-CTP for incorporation into DNA, thus inhibiting DNA polymerase. All these effects lead to termination of DNA elongation and finally to apoptosis (Faderl et al, 2002).

Another category of inhibitors inactivates RR by destroying the tyrosyl radical. These radical scavengers target R2 or p53R2 subunits (e.g. Hydroxyurea or Trimidox) (Lassmann et al, 1992; Szekeres et al, 1994).

Various studies proved that combinations of subunit-specific inhibitors result in synergistic inhibition of cell proliferation. Such treatment plans may even avoid drug resistance of cancer cells (Szekeres et al, 1997).

Other investigations revealed the combination of genotoxic chemotherapeutic agents with specific-R2 subunit inhibitors as a promising treatment regimen (Strand et al, 2002; Cooperman; 2003).

## **2.2. Cancer**

### **2.2.1. Carcinogenesis - a multistep process**

Carcinogenesis describes the process of the transformation of normal cells into cancer cells. Cancer develops due to the loss of growth and proliferation regulation of cells. An aberrant cellular regulation is often influenced by exposure to carcinogens, which results in genetic damages. Two important groups of genes are involved in the on-set of cancer: tumor-suppressor genes normally cause the constraint of cell growth and cell survival while proto-oncogenes promote the growth. A third group named caretaker genes normally protect the genome integrity by repairing or preventing DNA damages (Molecular cell biology/Lodish et al (2008)-6<sup>th</sup> ed.; p.1107).

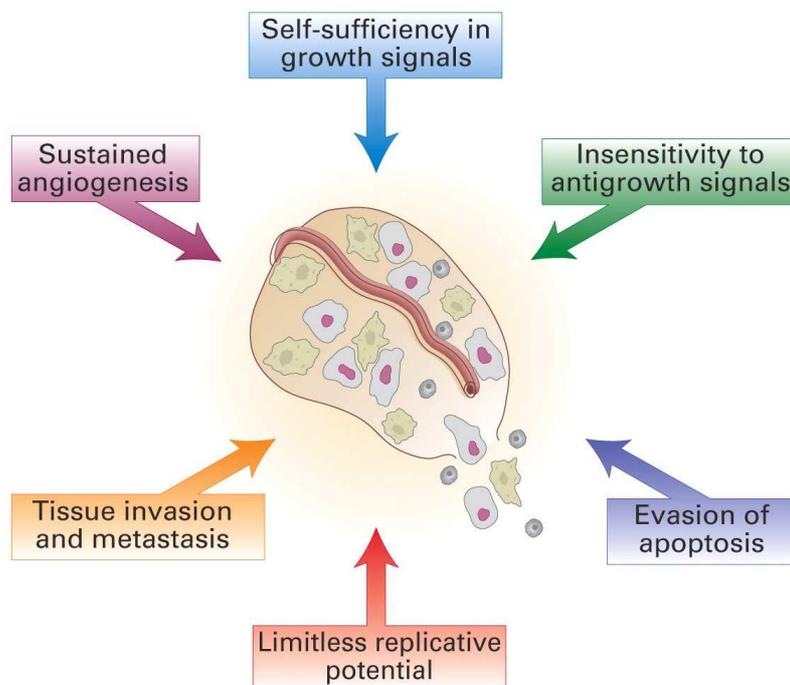
Loss-of-function mutations in tumor-suppressor genes or caretaker genes lead to unregulated cell proliferation or to accumulation of mutation while gain-of-function mutations transform proto-oncogenes into oncogenes allowing unregulated cell proliferation (Molecular cell biology/Lodish et al (2008)-6<sup>th</sup> ed.; 25.2).

The development of cancer requires an accumulation of mutations (Barrett, 1993), so cancers mainly establish later in life. Carcinogenesis is a multistep process and can be divided into three different periods: initiation, promotion and progression.

Initiation phase of carcinogenesis is characterized by an irreversible DNA damage being a result of extrinsic or intrinsic cytotoxic exposures. Quiescent initiated cells enter promotion-phase due to influence of non-carcinogenic compounds which results in clonal expansion of cells. Hennings et al revealed the reversibility of promoting effects, which seems to depend on epigenetic mechanisms (Hennings et al, 1993). Promotion phase develops a benign foci consisting of pre-neoplastic cells. The conversion of pre-neoplastic cells into malignant cancer cells is called progression. Benign tumors and malignant neoplasms essentially differ in morphology, invasiveness differentiation and growth (Barrett, 1993).

### 2.2.2. The hallmarks of cancer

Hanahan and Weinberg described six fundamental cellular properties which are altered during carcinogenesis (figure 2) (Hanahan and Weinberg, 2000). All these physiological alterations are responsible for the development of a destructive cancer phenotype.



**Figure 2: Six fundamental properties in cell physiology causing cancer (Molecular cell biology/ Lodish et al (2004)-5<sup>th</sup> ed.)**

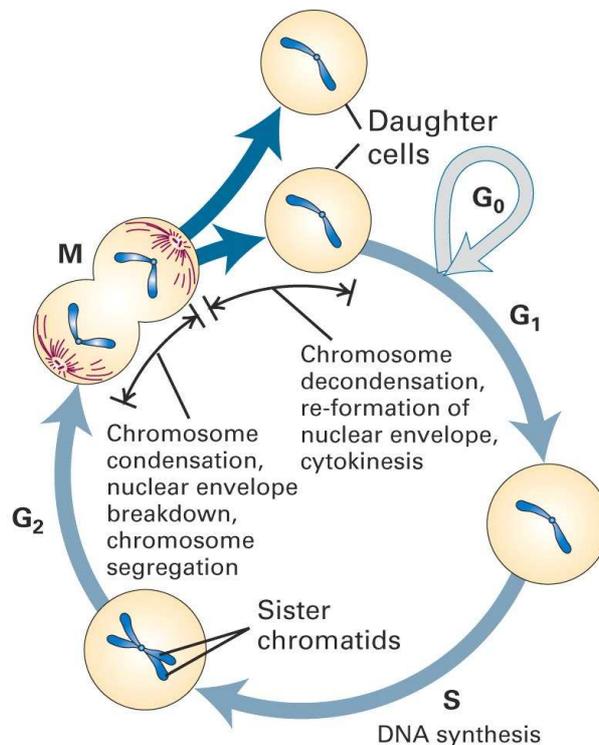
Cancer cells are proliferating without requiring external signals, they fail to sense cell cycle regulation, they are moving in other tissues and induce the development of blood vessels into the carcinogenic tissue (Molecular cell biology/Lodish et al (2008)-6<sup>th</sup> ed.; p.1108).

### 2.2.3. Cell cycle and Cancer

The term cell cycle describes serial events leading to subsequent cell division and two identical products. The cell cycle in mammalian cells is usually divided into four distinct phases: G<sub>1</sub> (gap phase 1), S (phase of synthesis), G<sub>2</sub> (gap phase 2), M (mitosis) (figure 3).

To achieve an accurate cell replication and segregation a multiple checkpoint control mechanism prevents abnormalities in cell cycle progression. Altered activation of these checkpoint-proteins may lead to the development of abnormal cell growth, differed gene expression levels and finally cancer cells (Meeran and Katiyar, 2008).

The whole preparation for cell division is done during the interphase that includes G<sub>1</sub>-, S- and G<sub>2</sub>-phase. Furthermore, cells can stop in G<sub>1</sub>, before DNA replication is started and pause in the so-called G<sub>0</sub> phase. Entering quiescent G<sub>0</sub> state depends on external and regulatory signals (van den Heuvel, 2005).



**Figure 3: Eukaryotic cell cycle (simplified) (Molecular cell biology/ Lodish et al (2008)-6<sup>th</sup> ed.)**

Chromosome replication occurs in the S-phase; that requires cell growth and DNA synthesis in the preceded  $G_1$ -phase. In the second gap  $G_2$ -phase, cells are preparing for mitosis in the following so called M-phase (Vermeulen et al, 2003).

#### **2.2.3.1. Cell cycle regulation by cyclin-dependent kinases (CDK)**

For coordinating the different events in the cell cycle, specific regulatory proteins are activated at specific moments of the division cycle. These controllers are heterodimeric cyclin-dependent kinases (CDKs). The expression level of CDKs is stable throughout the whole cell cycle, compared to the cyclins, the regulatory subunits of the kinases. Cyclin amounts in the cell differ during the division cycle, thereby affecting CDKs kinase activity. There are different types of cyclins for every single phase in the cell cycle. (Vermeulen et al, 2003).

CDKs are associated with specific cyclins and form cyclin-CDK complexes (Murray, 2004). These activated complexes regulate multiple downstream proteins involved in the cell cycle progression by phosphorylation (Morgan, 1995).

The cyclin-CDKs can be divided into three interphase kinases: CDK2, CDK4, and CDK6. Furthermore one mitotic CDK is known; the so-called CDK1 or cdc2. Cell-cycle related cyclins include type A, B, D and E (Meeran and Katiyar, 2008).

Cyclins D and E contain specific protein sequences for efficient proteolysis at the end of every cycle phase (Rechsteiner and Rogers, 1996). Hence they can periodically activate the kinases.

CDKs can also be regulated by inhibitory proteins (CKI), which have high affinity for CDKs or cyclin-CDK complexes. Thus, CDKs are modulated by two different classes of inhibitors: the INK4 and Cip/Kip families. p16, a member of the INK4 family inactivates  $G_1$  CDKs, and forms a stable CDK-CKI complex preventing cyclin-D association (Vermeulen et al, 2003).

Deregulation of cyclin-CDK complexes and therefore disorder of the cell cycle results in permanent cell proliferation, which may lead to the development of cancer.

### 2.2.3.2. Cell cycle regulation by checkpoint proteins

Controlling the eukaryotic cell cycle, multiple layers of regulation mechanisms are involved. These additional building blocks of the regulatory system are termed checkpoint kinases. With that difficult control system, cells react on DNA damage by interrupting cell cycle progression or by apoptosis. Deregulation of the key regulatory molecules is associated with increased cancer risk (Meeran and Katiyar, 2008). In the following a short overview of the three major checkpoints is given:

#### *S-phase checkpoint*

The intra-S-phase checkpoint network ensures that broken or damaged DNA is recognized by the control system. An important pathway causes the activation of ATM/ATR-Chk1 cascade. According to the type of damage Chk1 is phosphorylated by ATM or ATR, leading to further phosphorylation and down regulation of the Cdc25A phosphatase (Kastan and Bartek, 2004). That inhibition inactivates the mitotic cyclin E-CDK2 complex, which is essential for proceeding in S-phase (Falck et al, 2002).

#### *DNA-damage checkpoint*

G<sub>1</sub>/S checkpoint kinases prevent copying of damaged DNA by arresting the cell cycle until the damaged bases are exchanged. Occurrence of DNA damages at that checkpoint seems to be a crucial step to the onset of cancer (Pinto et al, 2005; Sherr and Roberts, 1999). This checkpoint is mediated by the ATM/ATR-Chk1/Chk2-Cdc25A pathway. UV-damaged DNA activates ATM kinases which phosphorylates Chk2. Activated Chk2 phosphorylates Cdc25A, initiating its degradation. DNA damage due to  $\gamma$ -irradiation activates ATR and Chk1, which also leads to Cdc25A ubiquitination. The degradation of Cdc25A results in G<sub>1</sub>/S-arrest (Kastan and Bartek, 2004).

The p53 tumor suppressor represents another important regulatory protein in G<sub>1</sub> checkpoint. Normally p53 transcription factor is extremely unstable and rapidly degraded. Phosphorylation of p53 by ATM/ATR (Ser<sup>15</sup>) and by Chk1/Chk2 (Ser<sup>20</sup>) inhibits degradation, promotes accumulation, and causes arrest in G<sub>1</sub>/S phase. A key target of the transcription

factor p53 is a gene encoding for p21<sup>CIP</sup> - a CKI of the Cip/Kip family - that inhibits cyclinE/CDK2 kinase and leads to inhibition of G<sub>1</sub>/S transition until damaged DNA bases are repaired (Meeran and Katiyar, 2008).

Besides its regulating mechanism in the cell cycle, p53 can also activate the expression of genes initiating apoptosis. These ambivalent properties may provide the observations that in nearly all cancer cells the p53 gene is mutated in both alleles (Molecular cell biology/Lodish et al (2008)-6<sup>th</sup> ed.; p. 891).

### *G<sub>2</sub>/M-checkpoint*

G<sub>2</sub>/M-checkpoint proteins sense DNA damages during G<sub>2</sub>-phase or damages without proper repair in G<sub>1</sub>- or S-phase and prevent cells from initiating mitosis. Thereby mitosis promoting cyclinB/CDK1 (cdc2) kinase is inhibited by Chk1/Chk2 and/or by p38 via inhibition and degradation of Cdc25 phosphatase family proteins (Kastan and Bartek, 2004).

BRCA1 and p53 were discussed to be essential for the regulation of G<sub>2</sub>/M-checkpoint by targeting p21<sup>CIP</sup>, which causes dissociation of the cyclinB/CDK1 complex (Nyberg et al, 2002; Taylor and Stark, 2001).

## **2.2.4. Cell death programs**

Programmed cell death is an essential process in maintenance and formation of tissues, during immune regulation or homeostasis. An accurate regulatory system performs numerous events leading to fatal morphological changes and ultimately to death. Due to differences in regulation and morphology, programmed cell death can be divided into apoptosis, necrosis and autophagy.

### **2.2.4.1. Apoptotic cell death**

Apoptotic cells are marked by well-defined morphological changes: cells shrink, condense and fragment. Released apoptotic bodies are finally engulfed by phagocytes and surrounding cells.

The apoptotic regulatory system comprises two important protein families: on the one hand the Bcl-2 family, which controls mitochondrial integrity and on the other hand the caspases (cysteiny l aspartate-specific proteases), which execute the apoptotic process (Youle and Strasser, 2008; Fuentes-Prior and Salvesen, 2008).

Human apoptotic caspases are homodimers and can be divided into executioner (caspase-3, -6, -7) and initiator (caspases-2, -8, -9, -10) caspases. Proteins are initially synthesized as procaspases and proteolytically cleaved into the active protein. Among other intrinsic or extrinsic regulatory pathways, the Bcl-2 family regulates processing of the proproteins by antagonizing pro- (e.g. Bax) and anti- (e.g. Bcl-2) apoptotic Bcl-2 family members. Thereby the Bcl-2 family controls the release of mitochondrial cytochrome c. Released cytochrome c binds to Apaf-1 (apoptotic protease activating factor) and starts and initiates the caspase cascade resulting in cell death (Duprez et al, 2009).

#### **2.2.4.2. Necrotic cell death**

Necrotic cell death usually results from tissue damage and seems to be an accidental and uncontrolled cell death. Krysko et al characterized necrosis by the absence of cytochrome c release, proteolytic processing of caspases and DNA fragmentation (Krysko et al, 2008). That process exhibits specific morphological changes: cells are swelling and bursting, and release their intracellular content, which provokes inflammatory reactions.

Investigations showed that in certain conditions strictly regulated pathways might promote necrosis, initiated by various stimuli. Necrosis may act as a back up for apoptotic cell death: hampering caspase pathways, necrosis may ensue instead (Duprez et al, 2009).

#### **2.2.4.3. Autophagic cell death**

Autophagy describes a catabolic process by which cells degrade and recycle cellular components (e.g. aged organelles). Materials are enveloped by plasma membranes and the internalized components are degraded in lysosomes. Precursors of lysosomes are autophagosomes, which are provided with a double-membrane. In contrast to apoptosis the cytoskeleton in autophagic cells remains intact.

Massive autophagy can result in cellular dying, therefore a connection between autophagy and necrotic or apoptotic cell death may exist (Maiuri et al, 2007).

## 2.3. Pancreatic Cancer

Pancreatic cancer is a very aggressive malignant neoplasm of the pancreas. Exocrine tumors are the most common pancreatic carcinomas; about 95% of these pancreatic malignancies are adenocarcinomas. Endocrine tumors make up about 1% of total cases (Yao et al, 2007).

### 2.3.1. Epidemiology and Etiology

#### *Epidemiology*

Approximately 270,000 new cases of pancreatic cancer occurred worldwide in 2008 (<http://info.cancerresearchuk.org>). Cancer of the pancreas is the fourth (entweder-oder!) most common cause of cancer death in Western countries. 80-90% of these cases are diagnosed in the non-resectable stage (Rosewicz et al, 1997; Yeo et al, 1999). Therefore, pancreatic cancer has a poor prognosis and its survival rate beyond five years is only about 3% (Niederhuber et al, 1995).

Cancer of the pancreas occurs in over 80% of cases in individuals between 60 and 80 years of age (Gold et al, 1998). Furthermore, it is more common in males than in females, possibly due to different lifestyles or occupational risk factors (Ahlgren et al, 1996). The highest incidence of pancreatic cancer in men has been detected in New Zealand Maoris and American black populations (Boyle et al, 1989).

#### *Etiology*

The causes of pancreatic cancer are ranging from cystic fibrosis – caused by a genetic mutation – to purely environmental diseases. Two large studies (Lichtenstein et al, 2000) showed that environmental factors are the most important causes of most types of cancer.

Smoking is the most common known risk factor causing about 25% of all pancreatic tumors. Compared to non-smokers smokers have a two-fold increased risk of pancreatic cancer. Numerous epidemiological studies revealed a significant correlation between the duration and intensity of cigarette smoking and the risk of pancreatic cancer (Silverman et al, 1994; Doll et

al, 1994; Lin et al, 2002). Furthermore, it is reported that the risk of pancreatic cancer in smokers is second only to lung cancer in men and women (Neuget et al, 1995).

In 2003, Calle et al showed in a cohort study of US adults that caloric consumption and/or obesity could be a risk factor for pancreas cancer. High intake of cholesterol and animal fat increases the risk, while intake of dietary fibre and Vitamin C decreases the risk of pancreatic cancer.

Several studies revealed an association of chronic pancreatitis and long-standing type II diabetes with an increased risk of pancreatic cancer (Lowenfels et al, 1999; Huxely et al, 2005).

The role of alcohol in the etiology of pancreatic cancer was investigated by several studies. However, most studies failed to establish a link between alcohol intake in moderate amounts and increasing pancreatic cancer risk (Lowenfels et al, 2006; Lin et al, 2002).

In recent years epidemiological studies have confirmed that several inherited disorders are linked with pancreatic cancer. Pancreatic cancer is seen in some breast cancer families with *BRCA1* and *BRCA2* mutations (Johansson et al, 1996; Phelan et al, 1996). Epidemiological studies have confirmed that people with a family history of pancreatic cancer have a higher risk of this malignancy (Ghadirian et al, 2003).

Some studies assumed that people infected with *Helicobacter pylori* - listed as a proven carcinogen by the International Agency for Research on Cancer - appeared to be at high risk of pancreatic cancer. However, the association between pancreatic cancer and *H. pylori* infection is not regarded as proven (Jesnowski et al, 2010; Lowenfels et al, 2006).

### **2.3.2. Treatment**

Pancreatic cancer patients often do not experience symptoms such as weight loss, pain or jaundice. In these cases the diagnosis may not be suspected; therefore unfortunately only 10-20% of patients present resectable diseases at the time of diagnosis (Loos et al, 2008).

For 80-90% of patients, chemotherapy and/or radiotherapy remain the only treatment option(s). A study of Glimelius et al showed that chemotherapy could prolong survival, palliate symptoms and improve quality of life in about one-third of the patients. The average

survival rate of the chemotherapy group in the study was 2.5 to 6 months (Glimelius et al, 1996).

### *Surgical Treatment*

Radical surgical resection may be suggested as a curative or as a palliative treatment to improve the patient's quality of life. A surgery remains the only treatment offering an advantage in terms of overall survival (5-year survival range, 15-25%) (Di Marco et al 2010; Yeo et al, 2002)

The first successful resections (pancreatoduodenectomy or Whipple procedure) were performed by Walter Kausch in 1912 and Allan Whipple in 1934 (Whipple et al, 1935; Pugliese et al, 2008; Strasberg et al, 1997). Due to new advances in technology and techniques during the last decades, laparoscopic surgeries have opened new gates in a range of applications in patients with pancreatic diseases. The first laparoscopic pancreatoduodenectomy was described in 1994 (Gagner et al, 1994; Gagner et al, 1997; Dulucq et al, 2005).

The Whipple procedure involves the removal of the gallbladder, common bile duct, part of the duodenum and the head of the pancreas.

### *Radiation therapy*

Radiotherapy for pancreatic carcinoma is normally performed as chemoradiotherapy (CRT). Several randomized controlled trials showed that adjuvant or additive CRT prolongs survival by improved local tumor control (Kaiser et al, 1985).

Moertel et al already demonstrated in early studies that combined CRT followed by additive chemotherapy was superior to radiotherapy alone (Moertel et al, 1981; Moertel et al, 1961).

### *Chemotherapy*

Most patients with advanced pancreatic cancer cannot have their tumors removed surgically. In that case, palliative chemotherapy is given to improve quality of life, prolong survival and to reduce the size of the tumor.

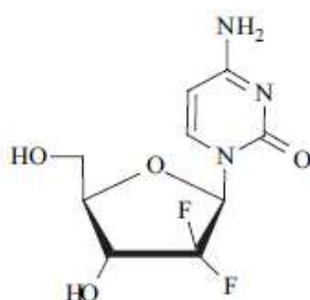
### *5-Fluorouracil*

Although pancreatic cancer cells are mainly resistant to chemotherapy, 5-fluorouracil (5-FU) has been shown to be superior to no treatment arms in adjuvant therapy trials. In several studies it has also been shown that various combination regimens with 5-FU achieve a modest benefit compared to no treatment arms in palliative therapy trials (Neoptolemos et al, 2003; Bakkevold et al, 1993). These combination regimens did not show any significant survival benefit compared with single-agent 5-FU (Cullinan et al, 1990).

5-FU is an S-phase-specific, fluorinated pyrimidine that is metabolized intracellularly to its active form fluorodeoxyuridine monophosphate (FdUMP) via the *de novo* pyrimidine pathway (Malet-Martino et al; 2002).

### *Gemcitabine*

During the 1990s multiple clinical studies have demonstrated the benefit of gemcitabine (dFdC) for pancreatic cancer compared with 5-FU (Sultana et al, 2010; Burris et al, 1994). Gemcitabine (2',2'-difluorodeoxycytidine; a pyrimidine metabolite; figure 4) is an S-phase nucleoside cytidine analogue and is involved in three different mechanisms: it competes for incorporation into DNA, it prevents DNA repair and it undergoes self-potentialiation (Huang et al, 1991).



**Figure 4: Structure of Gemcitabine**

Since its approval for use in 1996, dFdC is indicated as first-line treatment for patients with advanced pancreatic cancer. Phase I and II studies have demonstrated a 5-11% response rate

for advanced pancreatic cancer. Gemcitabine produced a median survival rate of 5.7-6.3 months (Burris et al, 1997; Carmicheal et al, 1996).

Recent phase I and II trials showed the effectiveness of the dFdC fixed-dose rate regimen (FDR) (Gelibter et al, 2005). However, a confirmatory phase III trial failed to show an improvement in the overall survival of the FDR regimen (Poplin et al, 2006). Furthermore, recent phase II studies demonstrated the efficacy of dFdC-combined regimens, but none of the randomized phase III studies confirmed an improvement of the overall survival compared to dFdC alone (Banu et al, 2007).

To sum up, it can be said that neither dFdC nor dFdC in combination with various drugs (5-FU, capecitabine, platinum compounds) have significantly improved the prognosis. The optimal chemotherapeutic drug or dosing regimen has not been established yet.

Recent investigations examined new molecular-targeted therapies alone or in combination with cytotoxic agents. Epidermal growth factor receptor (EGFR) and HER2/neu in pancreatic cancer have important key roles in the activation of main signaling pathways. All these pathways lead to activation of genes involved in apoptosis, cell proliferation and differentiation. EGFR and HER2/neu are frequently overexpressed in solid neoplasms including human pancreatic carcinoma. Therefore targeting the dysregulation of these transmembrane proteins with monoclonal antibodies directed against the extracellular ligand-binding domain might exert an antitumor effect (Potti et al, 2003; Yamanaka et al, 1993).

It is anticipated that further investigations will test new target biological agents or new combinations of dFdC. Cui et al showed that resveratrol, a phytoalexin inhibits the proliferation of pancreatic cancer cells (Cui et al, 2010). These results are in agreement with another previous study by Bernhaus et al indicating that KITC, a new resveratrol derivate, shows antitumor effects in human pancreatic cells (Bernhaus et al, 2009).

### **2.3.3. *In vitro* cell systems**

#### *AsPC-1*

The human AsPC-1 cell line was derived from nude mouse xenografts initiated with cells from the ascites of a patient with pancreatic cancer. The patient was a 62-year-old Caucasian woman with an adenocarcinoma in the pancreas (<http://www.atcc.org>).

*BxPC-3*

The human BxPC-3 cell line was derived from a 61-year-old female patient with an adenocarcinoma in the pancreas. The cells do not express the cystic fibrosis transmembrane conductance regulator (CFTR) (<http://www.atcc.org>).

*PANC-1*

The human PANC-1 cell line was derived from a 56-year-old male Caucasian patient with an epithelioid carcinoma in the pancreas (<http://www.atcc.org>).

## **2.4. The Leukemias**

### **2.4.1. Definition and Categorization**

Leukemia is cancer that starts in blood and bone marrow. It causes an abnormal increase of white blood cells, which enter the bloodstream. Leukemia, the most common blood cancer, is part of a group of diseases called hematological neoplasms.

Normally cellular blood components derive from pluripotent hematopoietic stem cells. The development of mature peripheral blood cells requires differentiation and a controlled proliferation of stem cells. Approximately  $10^{11}$ - $10^{12}$  new white blood cells, red blood cells and platelets are produced daily.

Leukemia is the result of certain mutations in the DNA. These genetic events cause activation of oncogenes or deactivation of tumor suppressor genes whereby progeny of the stem cells no longer differentiate and proliferate normally.

The leukemias can be grouped according to which kind of blood cell is affected (lymphoid versus myeloid) and according to the progress of disease. The acute leukemias are characterized by rapid increase of immature abnormal myeloid (in acute myeloid leukemia; AML) or lymphoid (in acute lymphoid leukemia; ALL) cells in the bone marrow. Due to the rapid uncontrolled accumulation and progression of the immature cells, symptoms in acute leukemias are quicker perceptible than in chronic leukemias. Malignant cells immigrate into the bloodstream and into other organs of the body. Symptoms include anemia, petechia, infection and bleeding ([www.cancer.gov](http://www.cancer.gov)).

Chronic leukemia is often found during a routine check-up because untreated people can live many years without any symptoms. It is characterized by an excessive proliferation of relatively mature blood cells. The still abnormal cells are accumulated in a higher rate than normal cells, resulting in the prevalence of abnormal white blood cells, which are genetically unstable. Due to the progression of the malignant clones, people get symptoms and the disease resembles acute leukemia. The chronic leukemias can also be subdivided into chronic myeloid leukemias (CML) and chronic lymphocytic leukemias (CLL).

## 2.4.2. Epidemiology and Etiology

### *Epidemiology*

Leukemia comprises about 3% of approximately seven million cancer deaths a year. It is the most common form of cancer among children age <15 years. Approximately 76% of all cases comprise acute lymphocytic leukemia whereas only 15-20% of diagnoses represent AML (Deschler and Lübbert, 2006). AML is therefore rather a disease for later adulthood with a median age of 65 years (Forman et al, 2003).

Over the past 20 years, a slight decrease in the incidence and mortality rates for leukemia was observed. Although there are remarkable improvements in the younger age group, the relative survival rate for older patients continue to be poor (Redaelli et al, 2003).

### *Etiology*

The different subtypes of Leukemia have different risk factors. The somatic mutations of the DNA, as discussed below, may be the result of exposure to a high level of (ionizing) radiation or carcinogenic substances. For example, Preston et al confirmed an excess of leukemia incidence and mortality in the group of Japanese atomic bomb survivors (Preston et al, 1994, 2004).

Ben-David et al showed that viruses such as RNA retroviruses cause neoplasms, including leukemia (Ben-David et al, 1991). The first identified human retrovirus, human T-lymphotropic virus type I (HTLV-I) is associated with lymphocytic leukemia (Bittencourt et al, 2006). However, it has not been demonstrated yet, that either a DNA- or RNA-based virus alone causes AML (Deschler and Lübbert, 2006).

Chronic exposure to chemicals, such as benzene, has been reported to increase the risk of developing AML (Savitz et al, 1997). Alkylating chemotherapeutic agents are also associated with an increased risk of AML (Le Beau et al, 1986).

Genetic or congenital disorders increase the risk of developing acute leukemia (Pui et al, 1995). Studies of twins have demonstrated this predisposition. Chromosomal abnormalities, such as Down syndrome have an increased likelihood of AML (Xavier and Taub, 2010).

### 2.4.3. Treatment

Normally Leukemia is diagnosed by an examination of the bone marrow or by the comparison of repeated blood counts. The aim of treatment is to accomplish complete remission, which means the complete regeneration of the bone marrow and the removal of leukemia cells from the blood. In most cases, the disease is treated with different regimens of multi-drug chemotherapy. Sometimes radiation therapy or bone marrow transplantations are applied to leukemia treatment ([www.cancer.gov](http://www.cancer.gov)).

#### *Acute Leukemia*

Initially, it is essential to differentiate between AML and ALL. In ALL, the standard chemotherapy regimen for adult comprises prednisolone, vincristine and an anthracycline. For children the drug therapy may include L-asparaginase. In children, the survival rate is close to 90% whereas in adults only 38% of patients can be cured (Pieters et al, 2011). The performance and the effectivity of bone-marrow transplantation remains a controversial discussed topic.

In AML, the cornerstone of induction therapy is the Ara-C. Standard-dose cytarabine for 7 days combined with an anthracycline (e.g. daunorubicin) for 3 days induces a complete remission in approximately 70% of patients (Robak et al, 2009).

#### *Chronic Leukemia*

The treatment of chronic leukemia rather focuses on alleviating symptoms than on healing up the disease. Treatment is normally started when symptoms and blood counts get worse and when quality of life is affected.

Currently, several effective combination chemotherapy plans showing a high response rate have been developed. Eichhorst et al reported that the combination regimen of anticancer drugs, such as fludarabine plus cyclophosphamide (FC), have longer progression-free survival (48 months) than treatment with single agents (20 months) (Eichhorst et al, 2006). A recent study supported the concept of synergistic combination chemoimmunotherapy of three agents.

Fludarabine and cyclophosphamide in combination with rituximab, a class I chimeric anti CD-20 antibody, revealed promising high complete remission rates (Keating et al, 2005).

Currently, there is no evidence that any described treatment regimens are curative. Patients relapse and afterwards develop resistance to the chemotherapeutic agents (Rai et al, 2003). Therefore, further approaches must take into account the balance between the increased risk for dying of stem-cell transplantation (SCT) in chronic lymphocytic leukemia and the potential to cure this disease (Gribben et al, 2008).

#### **2.4.4. In vitro cell system**

The human HL-60 is an acute promyelocytic leukemia cell line (APL) derived by Collins et al. Peripheral blood leukocytes were obtained from a 36-year-old Caucasian woman with acute promyelocytic leukemia (<http://www.atcc.org>).

HL-60 cells are representing a subtype of AML. This leukemia is a highly malignant neoplasm and characterized by an inhibition of granulocytic differentiation and by a chromosomal translocation causing a rearrangement of the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ). In over 98% of cases of APL the RAR $\alpha$  gene on chromosom 17 is fused on the promyelocytic leukemia gene (APL) on chromosom 15-translocation t(15; 17)(q21; q22). The RAR $\alpha$ -APL fusion protein binds to specific binding sites on DNA, stopping transcription and granulocytic differentiation (Vitoux et al, 2007). At the moment all-trans retinoic acid (RA) or arsenic trioxide are the mainstays for treatment of APL. These compounds target RAR $\alpha$ -PML hybrid protein and inhibit its effect on the DNA of tumor cells.

Recent preclinical studies evaluate new drugs, alone or in combination (e.g. with anthracyclines) to treat this disease (Vitoux et al, 2007).

## 2.5. ABNM-13

ABNM-13 (N-hydroxy-2-(anthracene-2-yl-methylene)-hydrazinecarboximidamide) is a novel N-Hydroxy-N'-aminoguanidine.

Modern drug design uses qualitative and quantitative structure-activity relationship (QSAR) studies as an approach to find relationships between chemical structures or structure-related properties and biological activities of distinct compounds. Based on the prediction of the best QSAR model, we synthesized 13 novel compounds (ABNM-1 to ABNM-13) with potential RR inhibitory capacities. Five of these agents were active in human HL-60 promyelocytic leukemia cells and ABNM-13 was chosen as lead substance because of its pronounced inhibitory effects.

ABNM-13 represents an unknown compound with ambitious expectations effecting in cancer cell systems.

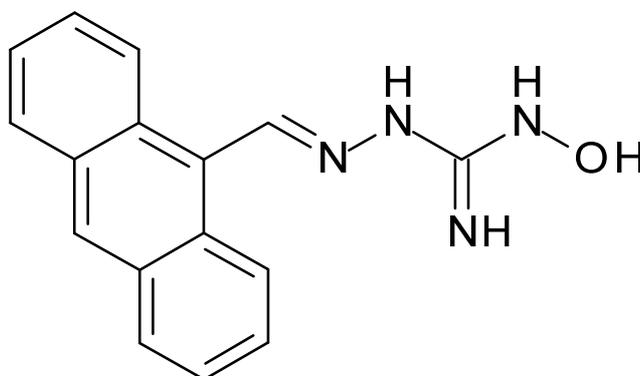
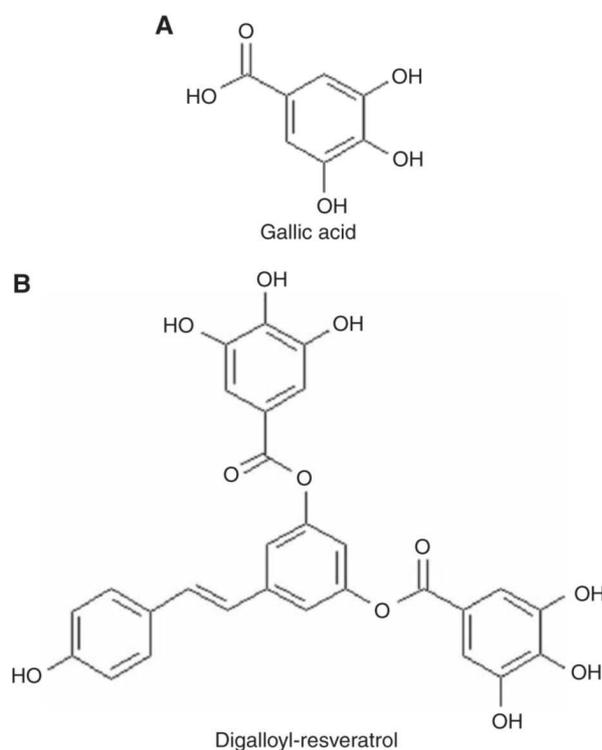


Figure 5: Structure of ABNM-13

## 2.6. DIG

Digalloylresveratrol (figure 6) (DIG; MW=532.47) is a novel synthetic ester of the polyhydroxy phenolic substances resveratrol (3,4,5-trihydroxystilbene; RV) and gallic acid (3,4,5-trihydroxybenzoic acid; GA). The compound was first synthesized and provided by Dr. Trimurtulu and co-workers, Laila Impex Research Center, Vijayawada 520 007, India (Gangaraju et al, 2006, US Patent # 7026518).

Gallic acid can be found in various natural products such as pineapples, strawberries, sumac, tea leaves, grapes, bananas, gallnuts, apple peels, lemons, red and white wine (De Beer et al, 2003; Sun et al, 2002; Wolfe et al, 2003). Resveratrol is a phytoalexin found in several plants, including mulberries, peanuts and grapes (Baur et al, 2006). Both naturally occurring compounds are probably responsible for a phenomenon known as the “French Paradox“. The latter is the fact that incidence of heart infarction in France is significantly lower (40%) than in other European countries or the USA (Renaud and De Lorgeril, 1992).



**Figure 6: Chemical structures of (A) gallic acid (GA) and (B) digalloylresveratrol (DIG) (Madlener et al, 2010)**

In Addition, the excellent radical scavenging activity of resveratrol and gallic acid was determined by several studies (Isuzugawa et al, 2001; Salucci et al, 2002; Horvath et al, 2005). It has been shown that RV and GA induce differentiation and programmed cell death in a variety of tumor cell lines (Kawada et al, 2001). Both compounds were identified as effective inhibitors of the enzyme ribonucleotide reductase (RR) (Madlener et al, 2007).

According to the promising cytotoxic effects of the single compounds, DIG was synthesized, consisting of two molecules gallic acid and one molecule resveratrol.

Bernhaus et al showed that a combination of resveratrol and gallic acid (ratio 1:2) inhibited the growth of HT-29 colon cancer cells less than DIG. Furthermore, it was demonstrated that DIG led to an inhibition of the RR (Bernhaus et al, 2009).

In fact, in HL-60 cells the inhibition of cell proliferation by DIG exceeded that of GA by 10-fold (Madlener et al, 2007). It was also demonstrated that the pro-apoptotic property of 10 $\mu$ M DIG is about 10-times higher than 20 $\mu$ M GA. DIG also showed a much higher apoptotic activity than RV. These results may support the conclusion that the RV backbones, to which the galloyl-residues are connected, are responsible for the determined synergistic effects (Madlener et al, 2010).

### 3. Materials and Methods

#### 3.1. Chemicals and supplies

**ABNM 1-13** were synthesized and provided by the Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, India. Structural formulas are shown in figure 1. **Cytarabine** (Ara-C), **Resveratrol** (3,4,5-trihydroxystilbene; RV) and solvent DMSO were obtained from Sigma-Aldrich GmbH, Vienna, Austria and were of highest purity available.

**Gallic acid** (3,4,5-trihydroxybenzoic acid; GA) was purchased from SIGMA, Vienna, Austria. **Gemcitabine** (dFdC) was obtained from ACTAVIS Generica, Austria.

**Digalloylresveratrol** (DIG) was a gift from Univ.-Prof. Mag. Dr. Walter Jäger, Department of Clinical Pharmacy and Diagnostics, University of Vienna.

All other chemicals and reagents used were commercially available and of highest purity.

The following reagents and materials were purchased as described below:

Ammoniumdihydrogenphosphate	VWR Merck, Vienna, Austria
Cytidine-2-14C (56 Ci/mmol)	SIGMA, St. Louis, MO
Sarcosine	SIGMA, St. Louis, MO
TRIS/ EDTA buffer	SIGMA, St. Louis, MO
Proteinase K	Roche, Vienna, Austria
Sodium acetate	Life Technologies, Paisley, Scotland
Phenol	SIGMA, St. Louis, MO
Chloroform	SIGMA, St. Louis, MO
RNAse A	Amersham Pharmacia, Vienna,
Phosphoric acid	VWR Merck, Vienna, Austria
Acetonitril	J.T. Baker
Trichloroacetic acid	SIGMA, St. Louis, MO
Freon	SIGMA, St. Louis, MO
Tri-n-octylamine	SIGMA, St. Louis, MO
Methylamine	SIGMA, St. Louis, MO
Sodium periodate	SIGMA, St. Louis, MO
L-Rhamnose	SIGMA, St. Louis, MO
Deoxyadenosine triphosphate	SIGMA, St. Louis, MO
Deoxycytosine triphosphate	SIGMA, St. Louis, MO
Deoxythymidine triphosphate	SIGMA, St. Louis, MO
Deoxyguanosine triphosphate	SIGMA, St. Louis, MO
Adenosine triphosphate	SIGMA, St. Louis, MO
Cytidine triphosphate	SIGMA, St. Louis, MO
Guanosine triphosphate	SIGMA, St. Louis, MO

Thymidine triphosphate	SIGMA, St. Louis, MO
Uridine triphosphate	SIGMA, St. Louis, MO
Cellpack buffer	Mueller, Vienna, Austria
Agarose Type VII	SIGMA, St. Louis, MO
Trypan blue stain	Life Technologies, Paisley, Scotland
Ethanol	SIGMA, St. Louis, MO
Hoechst dye 33258	SIGMA, St. Louis, MO
Propidium iodide	SIGMA, St. Louis, MO

### 3.2. Cell culture

#### 3.2.1. Devices

Laminar air flow	Dipl. Ing. W. Ehret GmbH, Emmendingen, Germany
Cytoperm2 incubator	Heraeus, Vienna, Austria
Olympus IMT-2 Inverse	Olympus, Vienna, Austria
CC-110 microcellcounter	SYSMEX, Kobe, Japan
pocH- 100i Hepatology analyzer	SYSMEX, Kobe, Japan
Hettich Rotanta Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany
Centrifuge 5415R	Eppendorf
Water Bath	Gesellschaft für Labortechnik mbH, Burgwedel,
PH-Meter	Metrohm, Switzerland
Mettler PJ 300/AT250 scales	Mettler, Vienna, Austria
Magnetic Stirrer	Framo, Eisenbach, Germany
Certoclav CV/CV-EL Autoclav	Certoclav, Vienna, Austria

#### 3.2.2. 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 Bovine Serum, Heat Inactivated

Penicillin/Streptomycin 10.000U/ml solution

L-Glutamin 200mM solution

Trypsin-EDTA 0.25% solution; Trypsin-EDTA 2.5% solution

Sodium Pyruvate 100mM

### 3.2.3. Cell lines

All tumor cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The human **HL-60** promyelocytic leukemia cells were grown in RPMI 1640 medium with L-Glutamine supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-Glutamine and 1% Penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria).

The **AsPC-1** and **BxPC-3** human pancreatic cancer cells were grown in RPMI 1640 Medium with GLUTAMAX supplemented with 10% heat inactivated fetal calf serum (FCS), 1% Sodium Pyruvate and 1% Penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria). The **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 at 37°C in a humidified atmosphere.

The attaching pancreatic cancer cells were grown in a monolayer culture using 25cm<sup>2</sup> tissue culture flasks and were periodically detached from the flask surface using 0.25 or 2.5% trypsin ethylene-diamine tetraacetic acid (trypsin-EDTA) solution.

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.

### 3.3. Growth inhibition assay

HL-60 cells ( $0.1 \times 10^6$  per ml) were seeded in 25cm<sup>2</sup> Nunc tissue culture flasks and incubated with increasing concentrations of drugs at 37°C under cell culture conditions. Cell counts and IC<sub>50</sub> values were determined after 24, 48, and 72 hours using the microcellcounter CC-110. Viability of cells was determined by trypan blue staining. Results were calculated as number of viable cells. All experiments were performed in triplicate.

### **3.3.1. Simultaneous growth inhibition assay**

HL-60 cells ( $0.1 \times 10^6$  per ml) were simultaneously incubated with various concentrations of novel compounds and cytostatic drugs commonly used in cancer treatment (Ara-C or dFdC) for 72 hours. After that period, cells were counted using a microcellcounter CC-110.

### **3.3.2. Sequential growth inhibition assay**

HL-60 cells ( $0.1 \times 10^6$  per ml) were first incubated with different concentrations of novel compounds for 24 hours. Then the first compound was washed out and cells were further exposed to various concentrations of commonly used cytostatic drugs (Ara-C or dFdC) for another 48 hours. After that period, cells were counted using a microcellcounter CC-110.

## **3.4. Clonogenic assay**

Attaching pancreatic tumor cells ( $1 \times 10^3$  perwell) were seeded in 24-well plates and allowed to attach overnight at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere. After 24 hours, the medium was removed and replaced by a fresh medium containing the designated concentration of drugs. The plates were then incubated for 7 days. Subsequently, the medium was carefully removed from the wells and the plates were stained with 0.5% crystal violet solution for 5 min. Colonies of  $>50$  cells were counted using an inverted microscope at 40-fold magnification. All experiments were performed in triplicate and repeated three times.

### **3.4.1. Simultaneous clonogenic assay**

Pancreatic cells ( $1 \times 10^3$  per well) were plated in 24-well plates and allowed to attach overnight at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere. After 24 hours, the medium was removed and replaced by a fresh medium containing the designated concentrations of novel compounds and cytostatic drugs commonly used in cancer treatment (Ara-C or dFdC). The plates were then incubated for 7 days. Further procedure as described above.

### **3.4.2. Sequential clonogenic assay**

Pancreatic cells ( $1 \times 10^3$  per well) were plated in 24-well plates and allowed to attach overnight at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere. After 24 hours, the medium was removed and replaced by a fresh medium containing the designated concentration of the novel compound and incubated for another 24 hours. Then the medium was removed again and cells were further exposed to various concentrations of a commonly used cytostatic drug. The plates were then incubated for another 6 days. Further procedure as described above.

### **3.5. Hoechst dye 33258 and propidium iodide double staining**

Cells ( $0.4 \times 10^6$  per ml) were seeded in  $25\text{cm}^2$  Nunc tissue culture flasks and exposed to increasing concentrations of drugs for 24 or 48 hours. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) were added directly to the cells to final concentrations of  $5\mu\text{g/ml}$  and  $2\mu\text{g/ml}$ , respectively. After 60 min of incubation at  $37^\circ\text{C}$ , cells were examined on a Leica DMR XA fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters for Hoechst 33258 and PI. The Hoechst dye stains the nuclei of all cells and therefore allows to monitor nuclear changes associated with apoptosis, such as chromatin condensation and nuclear fragmentation. PI, on the other hand, is excluded from viable and early apoptotic cells; consequently, PI uptake indicates loss of membrane integrity characteristic of necrotic and late apoptotic cells. Therefore, this method allows to distinguish between early apoptosis, late apoptosis, and necrosis. Cells were photographed with a COHU high performance CCD camera (COHU Inc., San Diego, CA, USA) using Leica Q-fish software and were counted under a microscope and the number of apoptotic cells was given as percentage value.

### **3.6. Cell cycle distribution analysis**

Cells ( $0.4 \times 10^6$  per ml) were seeded in  $25\text{cm}^2$  Nunc tissue culture flasks and incubated with increasing concentrations of drugs at  $37^\circ\text{C}$  under cell culture conditions. After 24 hours, cells were harvested and suspended in 5ml cold PBS, centrifuged, resuspended and fixed in 3ml

cold ethanol (70%) for 30 min at 4°C. After two washing steps in cold PBS, RNase A and propidium iodide were added to a final concentration of 50µg/ml each and incubated at 4°C for 60 min before measurement. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

### **3.7. Determination of deoxyribonucleoside triphosphates (dNTPs)**

Logarithmically growing HL-60 or AsPC-1 cells were incubated with increasing concentrations of drugs for 24 hours. Afterwards,  $5 \times 10^7$  cells were separated for the extraction of dNTPs according to a method established Garrett et al. Cells were centrifuged at 1800 rpm for 5 min and then resuspended in 100µl phosphate-buffered saline. In this suspension, cells were lysed by addition of 10µl of trichloroacetic acid and the mixture was vortexed for 1 minute. The lysate was rested on ice for 30 min and then the protein was separated by centrifugation at 15000 rpm for 10 min in an Eppendorf microcentrifuge. The supernatant was removed and neutralized by adding 1.1 vol of Freon containing 0.5 M tri-n-octylamin. Aliquots of 100µl were periodated by adding 30µl of 4M methylamine solution and 10µl sodium periodate solution (concentration: 100g/l). After incubation at 37°C for 30 min, the reaction was stopped by adding 5µl of 1M rhamnose solution. The extracted dNTPs were measured using a Merck „La Chrom” HPLC system equipped with L-7200 autosampler, L-7100 pump, L-7400 UV detector, and D-7000 interface. Samples were eluted with a 3.2M ammonium phosphate buffer, pH 3.6 (pH adjusted by addition of 0.32mol/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.

### **3.7.1. Ara-CTP determination**

Ara-CTP determination was performed according to the same method without the periodation step and a 3.2M ammonium phosphate buffer, pH 3.3.

### **3.8. Incorporation of <sup>14</sup>C-labelled cytidine into DNA**

To analyze the effect of drug incubation on the activity of DNA synthesis, an assay was performed as described previously (Szekeres et al, 1994). Logarithmically growing HL-60 or AsPC-1 cells ( $0.3 \times 10^6$  cells per ml) were incubated with various concentrations of drugs for 24 hours. After the incubation period, cells were counted and pulsed with <sup>14</sup>C-cytidine (0.3125 $\mu$ Ci, 5nM) for 30 min at 37°C. Then cells were collected by centrifugation and washed with PBS. Total DNA was extracted from  $5 \times 10^6$  cells by phenol-chloroform-isoamyl alcohol extraction and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (PerkinElmer, Boston, MA).

### **3.9. MTT chemosensitivity assay**

AsPC-1 or HL-60 cells ( $5 \times 10^3$  per well) were seeded in 96-well microtiter plates in supplemented RPMI 1640 medium. AsPC-1 cells were allowed to attach overnight. Cells were then incubated with various concentrations of ABNM-13 for 96 hours at 37°C under cell culture conditions. After that period, the reduction of the yellow tetrazolium compound 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) by the mitochondrial dehydrogenases of viable cells to a purple formazan product was determined using an assay kit from Promega according to the supplier's manual. The change in absorbance at 550nm was tracked on a Wallac 1420 Victor 2 multilabel plate reader (PerkinElmer Life and Analytical Sciences). Drug effect was quantified as the percentage of control absorbance of reduced dye at this wavelength.

### 3.10. DPPH radical scavenging activity assay

The radical scavenging activity of the tested compounds was determined using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). In its radical form, DPPH absorbs at 515nm but upon reduction by an antioxidant or radical species, its absorption decreases. The reaction was started by the addition of 10 $\mu$ l of the compound (5–100 $\mu$ M final concentration) to 3.0ml of 0.1mM DPPH in methanol. The bleaching of DPPH was followed using an HP 8453 diode array spectrometer equipped with a magnetically stirred quartz cell. Absorbance was recorded for up to 10 min, although steady states of reaction were reached in most cases within 3 min. The reference cuvette contained up to 0.1mM DPPH in 3.0ml of methanol. The DPPH radical scavenging activity obtained for each compound was compared with that of ascorbic acid and  $\alpha$ -Tocopherol.

### 3.11. Western blotting

HL-60 cells or AsPC-1 cells were seeded in 25cm<sup>2</sup> tissue culture flasks or 100mm Tissue Culture Dishes, respectively, at a concentration of 2 $\times$ 10<sup>6</sup> per ml medium and incubated with a specific concentration of drug (IC<sub>50</sub> value) for 0.5, 2, 4, 8, and 24 hours.

After incubation cells were harvested, washed twice with ice-cold PBS (pH 7.2) and lysed in a buffer containing 150mM NaCl, 50mM Tris-buffered saline (Tris pH 8.0), 1% Triton X-100, 1mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (PIC; from a 100x stock). The lysate was centrifuged at 12000 rpm for 20 min at 4°C, and the supernatant was stored at -20°C until further analysis.

Equal amounts of lysate (protein samples) were loaded onto polyacrylamide gels. Proteins were electrophoresed (PAGE) at 120 volt (50mA) for approximately 1 hour and then electroblotted onto PVDF membranes (Hybond P, Amersham, Buckinghamshire, UK) at 95 V and 4°C for 80 min.

Equal sample loading was controlled by staining membranes with Ponceau S. After washing with PBS/Tween-20 (PBS/T) pH 7.2 or Tris/Tween-20 (TBS/T) pH 7.6, membranes were blocked for 60 min in blocking solution (5% non-fat dry milk in PBS containing 0.5% Tween-20 or in TBS containing 0.1% Tween-20).

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

Membranes were washed with PBS/Tween-20 (PBS/T) pH 7.2 or Tris/Tween-20 (TBS/T) pH 7.6. Chemoluminescence was developed by the ECL detection kit (Amersham, Buckinghamshire, UK) and then membranes were exposed to Amersham Hyperfilms.

### 3.11.1. Antibodies

chk1	#2345, polyclonal (Cell Signaling)
chk2	#2662, polyclonal (Cell Signaling)
pchk1	#2344, polyclonal (Cell Signaling)
pchk2	#2661, polyclonal (Cell Signaling)
cdc25A	#sc-7389, monoclonal (Santa Cruz)
cdc25B	#sc- 326, polyclonal (Santa Cruz)
cdc25C	#sc- 327, polyclonal (Santa Cruz)
pcdc25A (Ser75)	#ab47279, polyclonal (Abcam)
pcdc25A (Ser177)	#AP3046a, polyclonal Abgent)
cdc2	#sc-54, monoclonal (Santa Cruz)
pcdc2	#4539, monoclonal (Cell Signaling)
R1	#sc-11733, polyclonal (Santa Cruz)
R2	#sc-10848, polyclonal (Santa Cruz)
P53R2	#sc-10840, polyclonal (Santa Cruz)
anti-H2AX	#DR1017, polyclonal (Calbiochem)
p-ATM	#4526, monoclonal (Cell Signaling)
p-ATR	#2853, polyclonal (Cell Signaling)
Cleaved Caspase-3	#9661, polyclonal (Cell Signaling)
Anti-β-Actin	clone AC-15, mouse ascites fluid, No. A5441 (Sigma)
Phospho-p44/42 MAPK (Erk1/2)	#9106, monoclonal (Cell Signaling)
p44/42 MAPK (Erk1/2)	#4695, monoclonal (Cell Signaling)
anti-goat IgG antibody	#10209, (Santa Cruz)
anti-mouse IgG/ HRP	#P0260, polyclonal (Dako)
Anti-rabbit IgG/ HRP	#P0217, polyclonal (Dako)

### **3.12. Statistical calculations**

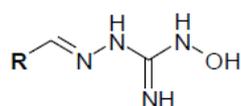
Dose-response curves were calculated using the Prism 5.01 software package (GraphPad, San Diego, CA, USA) and statistical significance was determined by unpaired t-test. The calculations of dose response curves and combination effects were performed using the "Calculusyn" software designed by Chou and Talalay (Biosoft, Ferguson, MO).

## 4. Results

### 4.1. ABNM-13

#### 4.1.1. Effect of ABNM 1-13 on the growth of HL-60 cells

HL-60 cells ( $0.1 \times 10^6$  per ml) were seeded in  $25\text{cm}^2$  Nunc tissue culture flasks and incubated with increasing concentrations of ABNM 1-13. After 72 hours, the cell number of viable leukemia cells was determined. ABNM-4, ABNM-8, ABNM-9, ABNM-12, and ABNM-13 inhibited the growth of HL-60 cells with  $\text{IC}_{50}$  values ( $\text{IC}_{50}$  = 50% growth inhibition of tumor cells) of 95, 67, 60, 62, and  $11\mu\text{M}$ , respectively. The  $\text{IC}_{50}$  values of all other compounds remained beyond  $100\mu\text{M}$  (figure 7).



ABNM	R	$\text{IC}_{50}$ ( $\mu\text{M}$ )
1		> 100
2		> 100
3		> 100
4		95
5		> 100
6		> 100
7		> 100
8		67
9		60
10		> 100
11		> 100
12		62
13		11

Figure 1: Structural and biological activity of ABNM 1-13 in HL-60 cells

All further investigations were established with the most efficacious compound, ABNM-13.

#### 4.1.2. Effect of ABNM-13 on the growth of HL-60 cells

HL-60 cells were seeded at a concentration of  $0.1 \times 10^6$  per ml and incubated with increasing concentrations of ABNM-13. After 24, 48 and 72 hours, the cell number of viable leukemia cells was determined. ABNM-13 inhibited the growth of HL-60 cells with  $IC_{50}$  values ( $IC_{50}$  = 50% growth inhibition of tumor cells) of 15 after 48 hours and  $11 \mu\text{M}$  after 72 hours, respectively (figure 8).

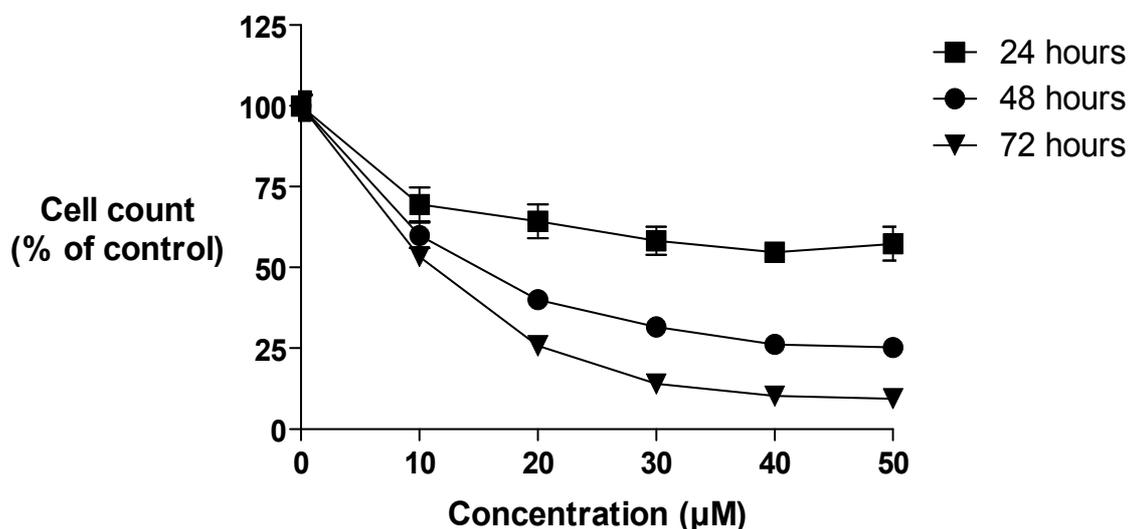


Figure 8: Growth inhibition of HL-60 cells after incubation with ABNM-13

##### 4.1.2.1. Synergistic combination effects of ABNM-13 and Ara-C in HL-60 cells

To investigate the effect of ABNM-13 in combination with Ara-C, HL-60 cells were seeded at a concentration of  $0.1 \times 10^6$  per ml and simultaneously or sequentially incubated with increasing concentrations of drugs (ABNM-13 first for 24 hours and then Ara-C for 48 hours as described in the methods section). Three out of twelve drug combinations caused synergism when ABNM-13 and Ara-C were applied simultaneously (table 1). Moreover, all twelve combinations yielded highly synergistic effects when applied sequentially (cells were first incubated with 2.5, 5, 7.5, and  $10 \mu\text{M}$  ABNM-13 followed by the addition of 5, 10, and 20nM Ara-C, respectively) (table 2).

**Table 1: Synergistic combination effects of ABNM-13 and Ara-C in HL-60 cells employing a simultaneous growth inhibition assay**

Compound	Concentration ( $\mu\text{M}/\text{nM}$ )	Cell number $\pm$ SD (% of control)	Predicted value*	Combination Index**
ABNM-13 (A) ( $\mu\text{M}$ )	2.5	96.4 $\pm$ 3.50		
	5.0	74.3 $\pm$ 0.64		
	7.5	68.1 $\pm$ 0.48		
	10.0	53.2 $\pm$ 2.23		
Ara-C (B) (nM)	10	62.0 $\pm$ 1.12		
	15	50.7 $\pm$ 0.96		
	20	40.8 $\pm$ 0.32		
ABNM-13 + Ara-C	2.5 10	55.2 $\pm$ 0.96	59.8	<b>1.064</b>
	2.5 15	39.8 $\pm$ 0.48	48.8	<b>0.924</b>
ABNM-13 + Ara-C	2.5 20	33.8 $\pm$ 0.64	39.3	<b>0.961</b>
	5 10	40.4 $\pm$ 1.12	46.1	<b>0.910</b>
ABNM-13 + Ara-C	5 15	31.9 $\pm$ 1.16	37.7	<b>0.899***</b>
	5 20	30.3 $\pm$ 2.70	30.3	<b>1.027</b>
ABNM-13 + Ara-C	7.5 10	3.1 $\pm$ 0.48	42.3	<b>1.005</b>
	7.5 15	28.8 $\pm$ 1.27	34.5	<b>0.824***</b>
ABNM-13 + Ara-C	7.5 20	26.4 $\pm$ 0.00	27.8	<b>1.057</b>
	10 10	30.3 $\pm$ 0.16	33.0	<b>1.031</b>
ABNM-13 + Ara-C	10 15	23.9 $\pm$ 0.32	26.9	<b>0.858***</b>
	10 20	22.6 $\pm$ 0.80	21.7	<b>1.074</b>

Cells were simultaneously incubated with (1) ABNM-13 for 24h and (2) Ara-C for 48h, and then the cell number was determined. Data are means of two determinations  $\pm$  standard deviations (SD).

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

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

\*\*\* Synergistic combination effect

**Table 2: Synergistic combination effects of ABNM-13 and Ara-C in HL-60 cells employing a sequential growth inhibition assay**

Compound	Concentration ( $\mu\text{M}/\text{nM}$ )	Cell number $\pm$ SD (% of control)	Predicted value*	Combination Index**
ABNM-13 (A) ( $\mu\text{M}$ )	2.5	88.7 $\pm$ 0.78		
	5.0	61.8 $\pm$ 0.16		
	7.5	50.9 $\pm$ 0.78		
	10.0	32.6 $\pm$ 0.31		
Ara-C (B) (nM)	10	72.4 $\pm$ 0.94		
	15	71.8 $\pm$ 3.61		
	20	59.3 $\pm$ 3.45		
ABNM-13 + Ara-C	2.5 10	49.2 $\pm$ 4.55	64.2	<b>0.607***</b>
	2.5 15	25.2 $\pm$ 4.87	63.7	<b>0.305***</b>
ABNM-13 + Ara-C	2.5 20	39.8 $\pm$ 2.35	52.6	<b>0.607***</b>
	5 10	15.5 $\pm$ 7.85	44.7	<b>0.329***</b>
ABNM-13 + Ara-C	5 15	20.0 $\pm$ 5.65	44.4	<b>0.418***</b>
	5 20	32.1 $\pm$ 1.10	36.6	<b>0.692***</b>
ABNM-13 + Ara-C	7.5 10	17.3 $\pm$ 5.02	36.9	<b>0.514***</b>
	7.5 15	26.4 $\pm$ 0.00	36.6	<b>0.740***</b>
ABNM-13 + Ara-C	7.5 20	23.2 $\pm$ 1.41	30.2	<b>0.695***</b>
	10 10	17.2 $\pm$ 2.04	23.6	<b>0.670***</b>
ABNM-13 + Ara-C	10 15	16.8 $\pm$ 1.73	23.4	<b>0.676***</b>
	10 20	17.9 $\pm$ 2.67	19.3	<b>0.727***</b>

Cells were sequentially incubated with (1) ABNM-13 for 24h and (2) Ara-C for 48h, and then the cell number was determined. Data are means of two determinations  $\pm$  standard deviations (SD).

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

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

\*\*\* Synergistic combination effect

#### 4.1.3. Inhibition of incorporation of $^{14}\text{C}$ -cytidine into DNA of HL-60 cells after treatment with ABNM-13 and/or Ara-C

The RR *in situ* activity was measured in HL-60 cells after incubation with increasing concentrations of ABNM-13. Exposure to 10, 20, and 40  $\mu\text{M}$  ABNM-13 for 24 hours significantly decreased  $^{14}\text{C}$ -cytidine incorporation into nascent DNA to 52%, 17%, and 4%, respectively (figure 9a).

To analyze the immediacy of RR inhibition, HL-60 cells were exposed to 15 nM Ara-C, 15  $\mu\text{M}$  ABNM-13, and the simultaneous combination of both compounds for only 30 min. Even this short incubation period reduced the incorporation of  $^{14}\text{C}$ cytidine to 93%, 27%, and 4% of controls, respectively (figure 9b).

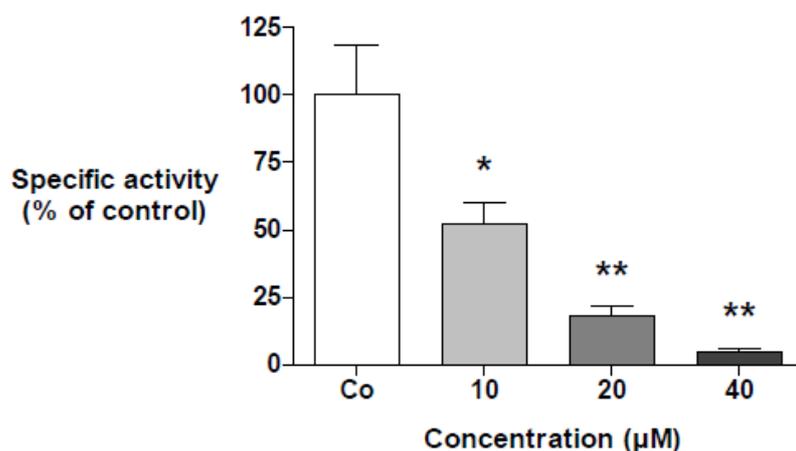


Figure 9a: Inhibition of  $^{14}\text{C}$ -incorporation into DNA of HL-60 cells after treatment with ABNM-13 for 24 hours

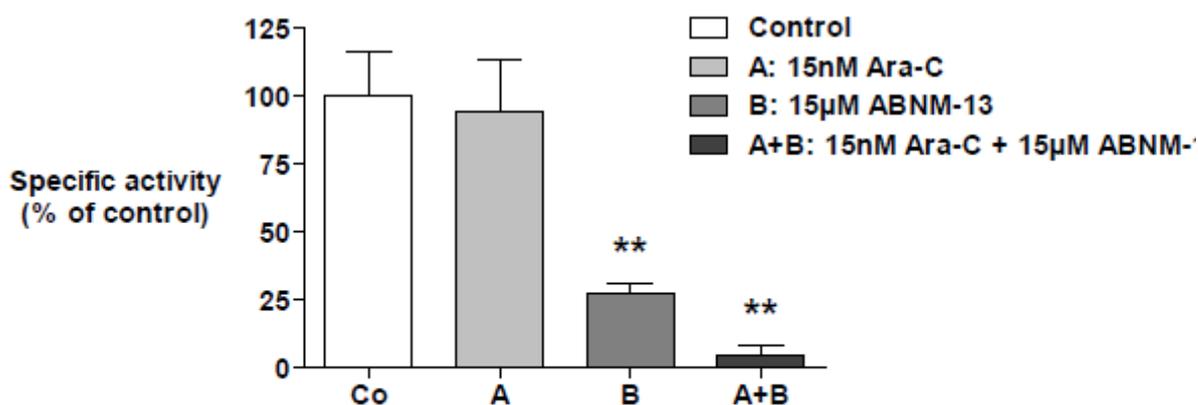
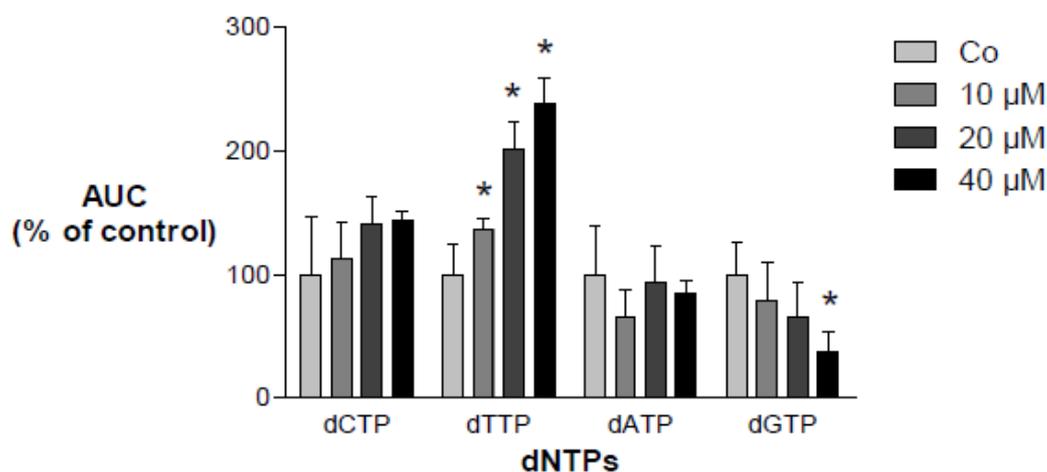


Figure 9b: Inhibition of  $^{14}\text{C}$ -incorporation into DNA of HL-60 cells after treatment with ABNM-13 and/or Ara-C for 30 min

#### 4.1.4. Alterations of dNTP pool sizes in HL-60 cells after treatment with ABNM-13 for 24 hours

Constitutive RR activity maintains balanced dNTP pools, whereas RR inhibition tilts this balance. In line with the inhibition of RR *in situ* activity, ABNM-13 treatment caused also an imbalance of dNTPs in HL-60 cells after 24 hours, which was determined by HPLC analysis. Incubation of cells with 40 $\mu$ M ABNM-13 resulted in a significant depletion of intracellular dGTP pools to 36%. Treatment with 10, 20, and 40 $\mu$ M ABNM-13 significantly increased dTTP pools to 134%, 200%, and 237% of control values, respectively. Regarding dCTP and dATP pools, treatment with ABNM-13 led to insignificant changes (figure 10).



**Figure 10: Concentration of dNTP pools in HL-60 cells after treatment with ABNM-13 for 24 hours**

#### 4.1.5. Expression of RR subunits R1, R2, and p53R2 after treatment with ABNM-13 and/or Ara-C

To monitor the effect of RR inhibitors on the expression of RR subunits, HL-60 cells were incubated with 15nM Ara-C and/or 15 $\mu$ M ABNM-13 for 0.5, 2, 4, 8, and 24 hours and subjected to western blot analysis. The protein level of the constitutively expressed R1 subunit remained unchanged during the whole time course, whereas the levels of S-phase specific R2 and p53R2 subunits were elevated after 8 and 24 hours of incubation (figure 11).

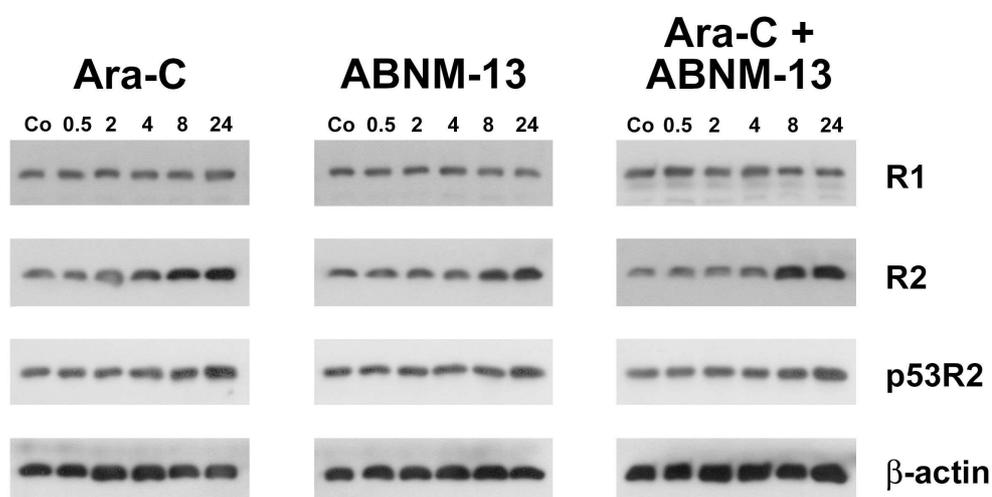


Figure 11: Expression of RR subunits in HL-60 cells after treatment with ABNM-13 and/or Ara-C

#### 4.1.6. Cell cycle distribution in HL-60 cells after treatment with ABNM-13 and/or Ara-C

HL-60 cells were simultaneously incubated with 15 $\mu$ M ABNM-13 and/or 15nM Ara-C for 24 hours. Treatment of HL-60 cells with 15 $\mu$ M ABNM-13 caused cell cycle arrest in S-phase, increasing this cell population from 34% to 62%, whereas G0-G1 phase cells decreased from 46% to 21%. 15nM Ara-C likewise caused an accumulation of 69% HL-60 cells in S-phase and a concomitant decrease of G0-G1 cells to 12%. Simultaneous incubation of HL-60 cells with 15 $\mu$ M ABNM-13 and 15nM Ara-C led to an even more pronounced growth arrest in the

S-phase, increasing this cell population from 34% to 94% while decreasing cells in the G0-G1 phase from 46% to 6% (figures 12a-c).

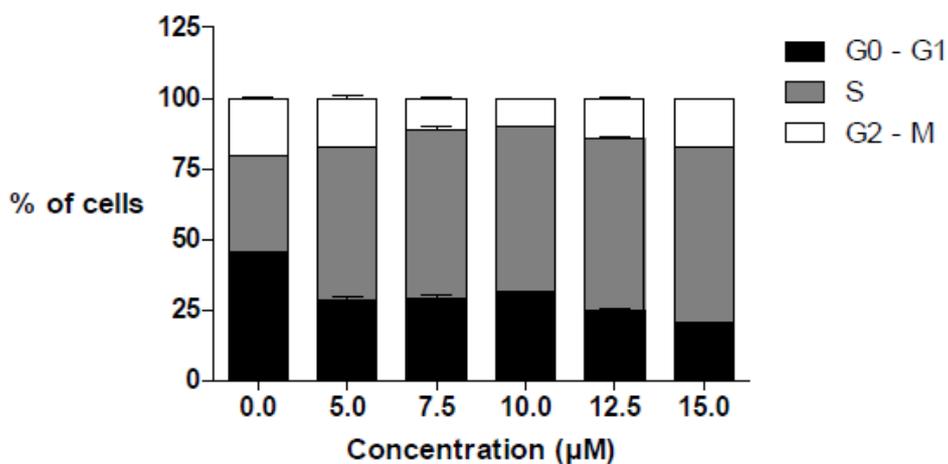


Figure 12a: HL-60 cell cycle distribution after incubation with ABNM-13

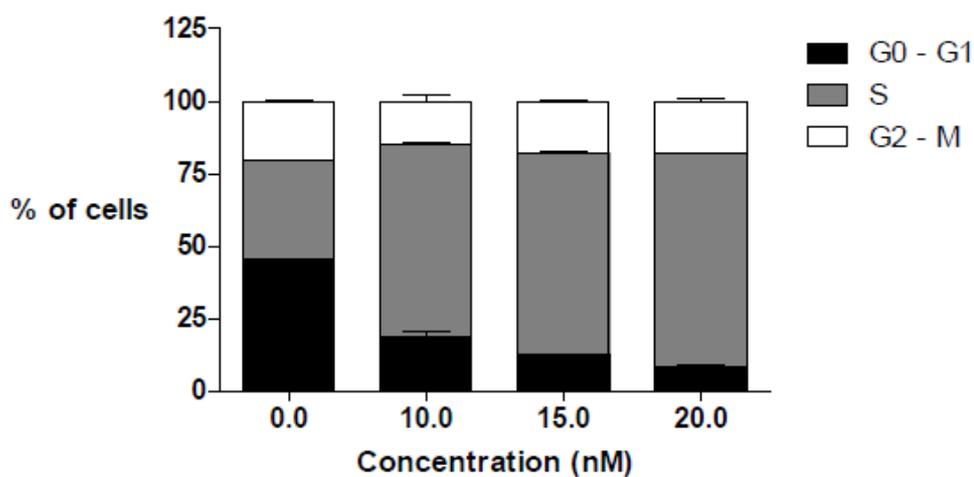


Figure 12b: HL-60 cell cycle distribution after incubation with Ara-C

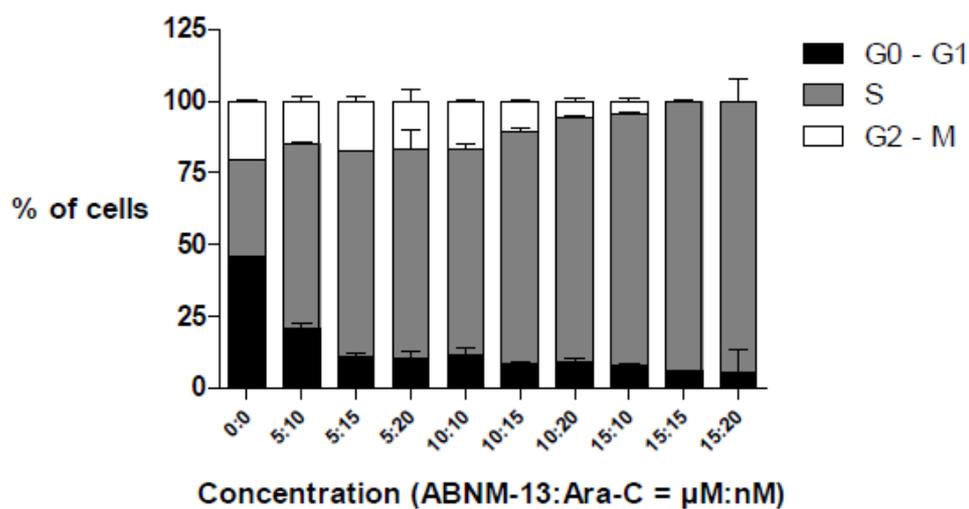
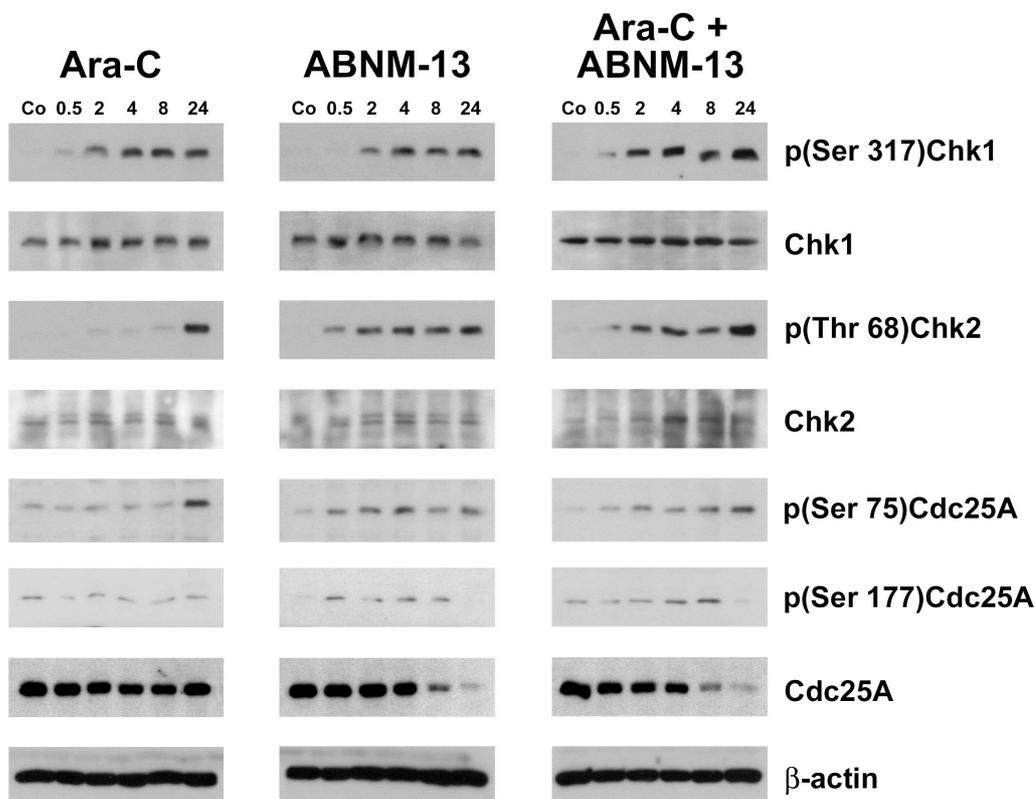


Figure 12c: HL-60 cell cycle distribution after simultaneous incubation with ABNM-13 and Ara-C

#### 4.1.7. Expression of checkpoint and cell cycle regulating proteins after treatment with ABNM-13 and/or Ara-C

To investigate whether S-phase inhibition caused activation of cell cycle checkpoint kinases, HL-60 cells were simultaneously treated with 15 nM Ara-C and/or 15  $\mu$ M ABNM-13 for 0.5, 2, 4, 8, and 24 hours and subjected to western blot analysis. Chk1 was phosphorylated at the activating Ser317 site within 30 min (Ara-C), 2 hours (ABNM-13), and 2 hours (Ara-C/ABNM-13). Chk2 was phosphorylated at the activating Thr68 site within 24 hours (Ara-C), 30 min (ABNM-13), and 30 min (ABNM-13/Ara-C). Chk1 and Chk2 protein levels remained unchanged (figure 13a). In addition, ABNM-13 caused phosphorylation at Ser75 and Ser177 of the dual-specificity phosphatase Cdc25A, which are target sites of Chk1 and Chk2, respectively, resulting in its downregulation after 8 and 24 hours. On the other hand, ABNM-13 upregulated Cdc25B protein levels after 24 hours (Ara-C after 8 and 24 hours), resulting in the dephosphorylation of Tyr15 of Cdk1 after 24 hours, which is indicative for its activation. Ara-C treatment did not cause dephosphorylation of Cdk1. Cdc25C levels remained unchanged throughout the time course (figure 13b).



**Figure 13a: Expression of checkpoint and cell cycle regulators upon treatment with ABNM-13 and/or Ara-C**

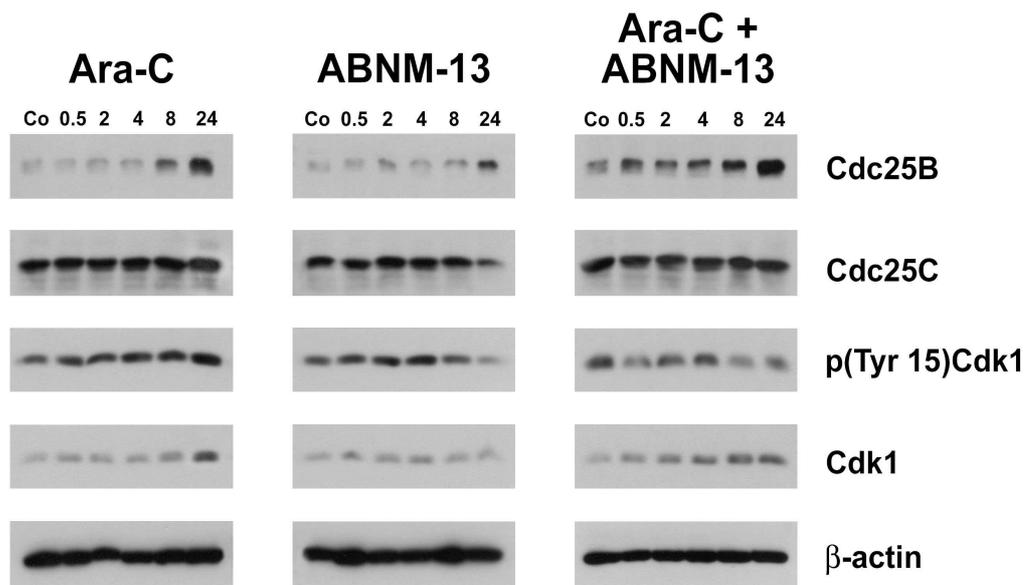


Figure 13b: Expression of cell cycle regulators upon treatment with ABNM-13 and/or Ara-C

#### 4.1.8. Induction of apoptosis in HL-60 cells by ABNM-13 and/or Ara-C

HL-60 cells were exposed to 12.5, 15, 17.5, and 20 $\mu$ M ABNM-13 for 48 hours and double stained with Hoechst 33258 and propidium iodide to analyze whether apoptotic cell death was induced. The nuclear morphology of 22% HL-60 cells showed early as well as late apoptosis stages upon treatment with 15 $\mu$ M ABNM-13 (figure 14a), indicating that cell cycle inhibition rather than induction of apoptosis seems to be the primary antineoplastic effect of ABNM-13. Incubation with 15nM Ara-C or the combination of 15 $\mu$ M ABNM-13 and 15nM Ara-C for 48 hours resulted in 10 and 28% apoptotic cells, respectively (figure 14b).

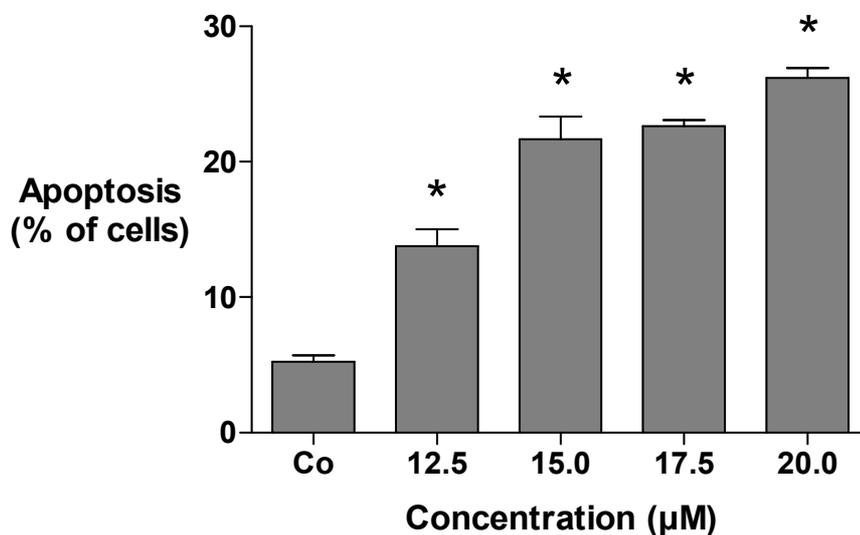


Figure 14a: Induction of apoptosis in HL-60 cells after treatment with ABNM-13

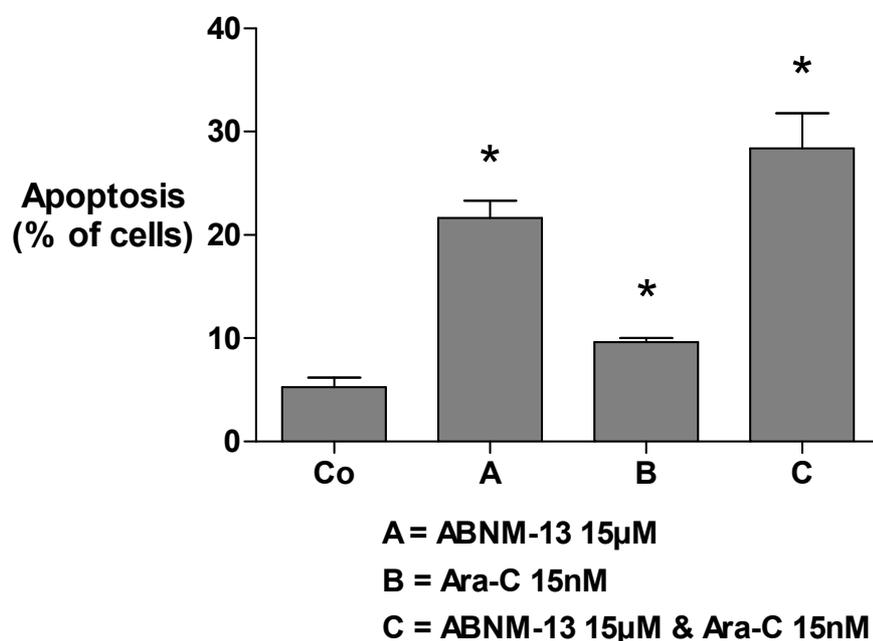
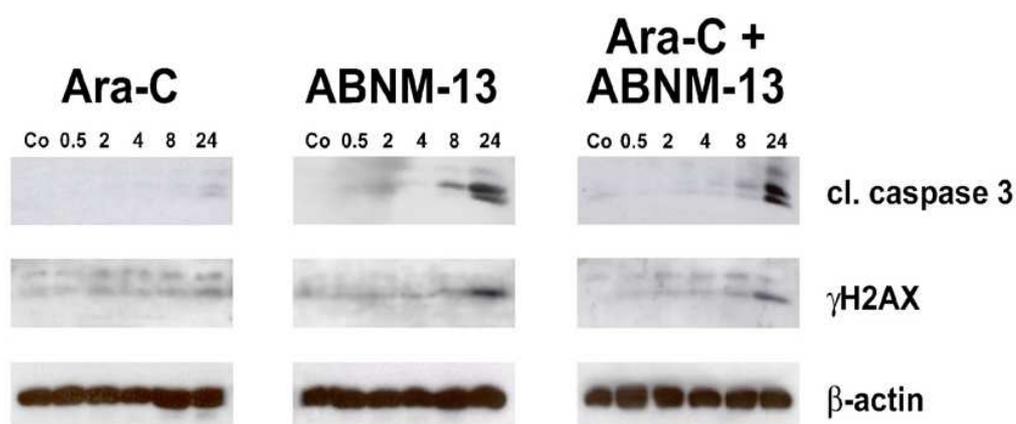
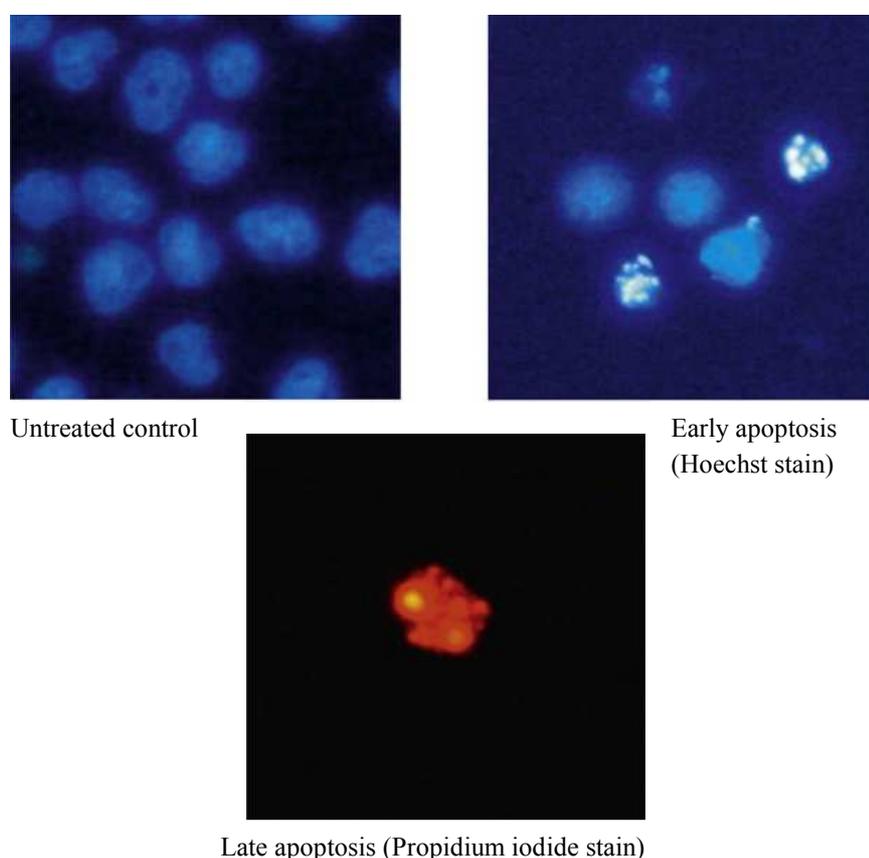


Figure 14b: Induction of apoptosis in HL-60 cells after treatment with 15 $\mu$ M ABNM-13 and/or 15nM Ara-C

The induction of apoptosis was further substantiated by the cleavage and therefore activation of caspase-3 after 8 and 24 hours of treatment with 15 $\mu$ M ABNM-13 or the combination of 15 $\mu$ M ABNM-13 and 15 nM Ara-C, which in turn led to increased protein levels of  $\gamma$ H2AX after 24 hours. In contrast, 15 nM Ara-C induced activated caspase-3 and  $\gamma$ H2AX levels only marginally (figure 14c).



**Figure 14c:** Expression levels of cleaved caspase-3 and  $\gamma$ H2AX after incubation with ABNM-13 and/or Ara-C



**Figure 14d:** Examples of the cellular morphology of HL-60 cell after incubation with ABNM-13 for 48 hours. Cells were double stained with Hoechst dye 33258 plus propidium iodide

#### 4.1.9. MTT chemosensitivity assay

HL-60 cells ( $5 \times 10^3$  per well) were seeded in 96-well microtiter plates and exposed to increasing concentrations of ABNM-13 as described in the methods section. After 96 hours of incubation, ABNM-13 reduced the absorbance (viability) of HL-60 cells with an  $IC_{50}$  value of  $9 \pm 1.7 \mu\text{M}$  (figure 15).

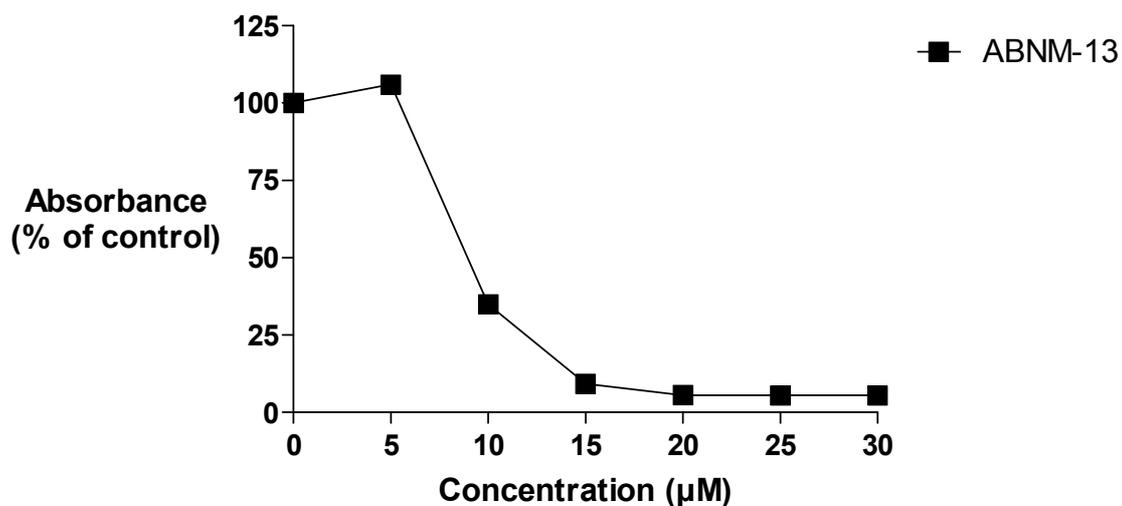
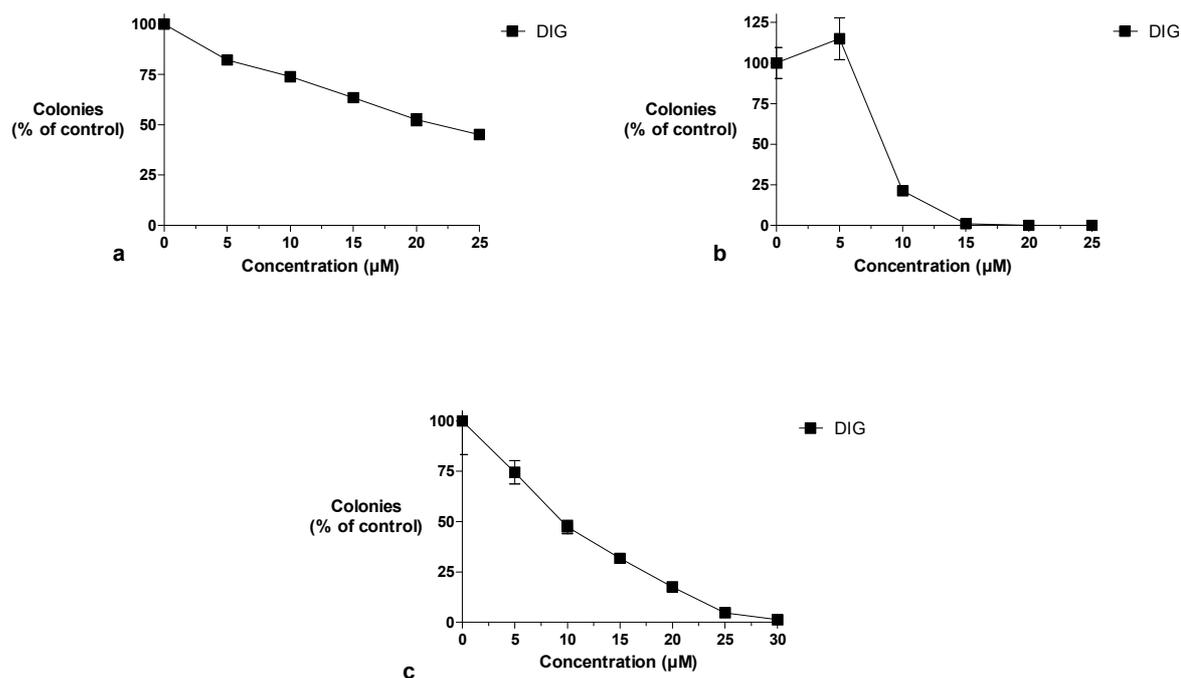


Figure 15: MTT chemosensitivity assay of HL-60 cells after treatment with ABNM-13

## 4.2. DIG

### 4.2.1. Clonogenic assays in AsPC-1, BxPC-3, PANC-1 cells – effects of DIG, dFdC, GA and RV

Logarithmically growing pancreatic cells were incubated with various concentrations of drugs for 7 days. Cell colonies were counted after 7 days of incubation as explained in the methods section. DIG inhibited the growth of AsPC-1 cell with an  $IC_{50}$  value of  $21.5\mu M$ , BxPC-3 cells with an  $IC_{50}$  value of  $8.5\mu M$  and PANC-1 cells with an  $IC_{50}$  value of  $9.5\mu M$  (figures 16a-c). All further inhibitory effects are showed in table 2.



Figures 16a-c: Growth inhibition of AsPC-1 (a), BxPC-3 (b), PANC-1 (c) cells after treatment with various concentrations of DIG

**Table 2: Comparison of the effect of DIG, dFdC, GA and RV on human pancreatic cancer cells.**

<b>Compound</b>	<b>MW</b>	<b>AsPC-1</b>	<b>BxPC-3</b>	<b>PANC-1</b>
<b>dFdC</b>	263.20	3.25nM	1.85nM	1.5nM
<b>DIG</b>	532.47	21.5µM	8.5µM	9.5µM
<b>GA</b>	170.12	21.0µM	41.0µM	8.5µM
<b>RV</b>	228.25	18.0µM	13.3µM	7.5µM

#### **4.2.1.1. Combination effects of DIG and dFdC on the growth of AsPC-1 cells**

To investigate the effect of DIG in combination with dFdC, AsPC-1 cells were seeded at a concentration of  $1 \times 10^3$  cells/well and sequentially incubated with increasing concentrations of drugs. Cells were first incubated with 25, 30, 35µM ABNM-13 for 24 hours and then with 2.5, 3, 3.5nM dFdC for 7 days as described in section 2. However, none of the 12 concentrations yielded any synergistic effects (data not shown).

#### 4.2.2. Antioxidant activity of DIG, RV, GA

The *in vitro* free radical-scavenging activity of DIG, RV, GA and equimolar combinations of RV and GA was determined employing a DPPH-assay. After incubation for 10 min DIG, RV, and GA inhibited 50% of DPPH activity with  $IC_{50}$  values of 1.83, 98.3, and  $3.12\mu\text{M}$ , respectively (figures 17a-d). The combination of RV and GA inhibited 50% of DPPH activity at  $4.82\mu\text{M}$ . Tocopherol and ascorbic acid were used as reference compounds resulting in  $IC_{50}$  values of  $6.98\mu\text{M}$  and  $9.63\mu\text{M}$ , respectively.

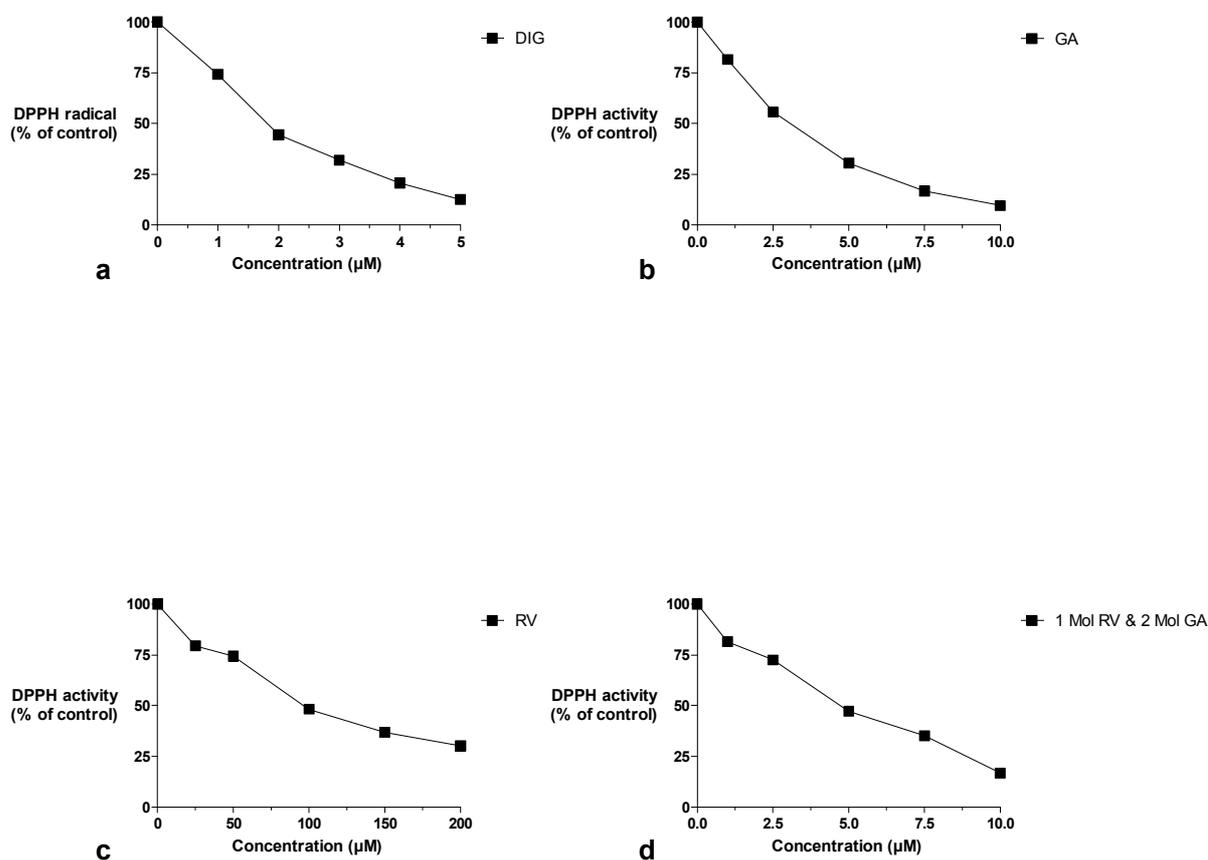
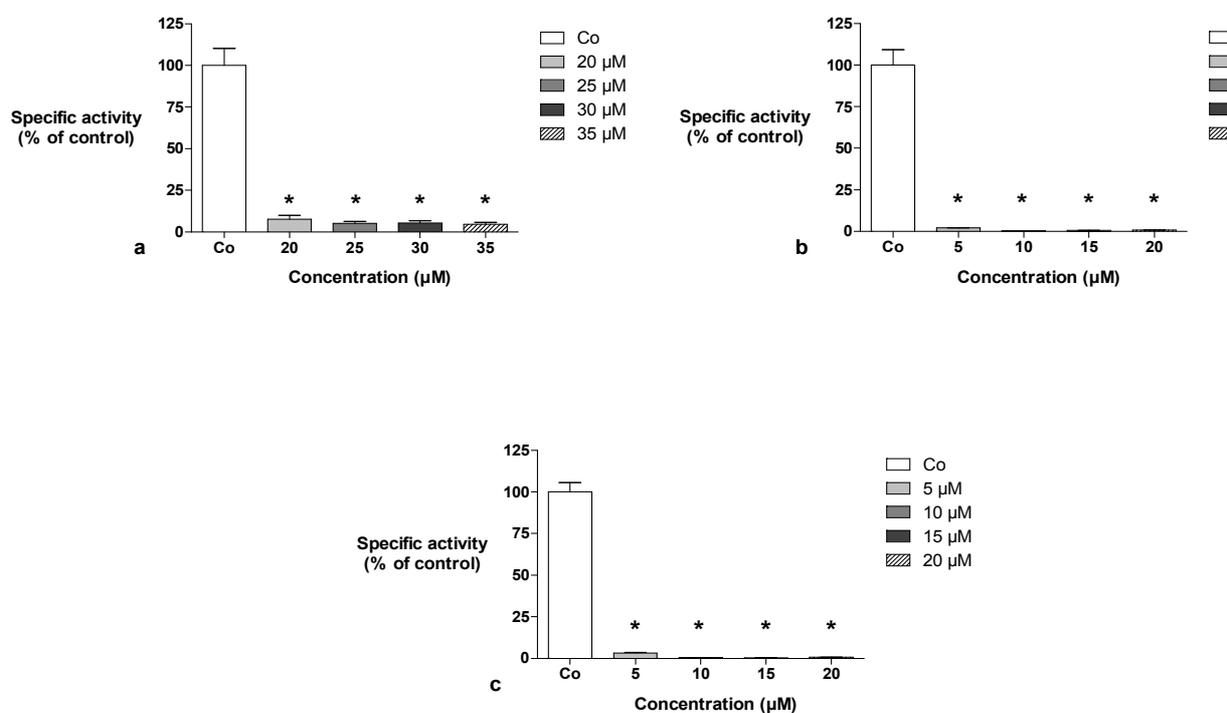


Figure 17: Antioxidant activity of DIG (a), GA (b), RV (c) and RV: GA (1:2) (d) after incubation for 10 min

#### 4.2.3. Inhibition of incorporation of $^{14}\text{C}$ -cytidine into DNA of AsPC-1, BxPC-3, PANC-1 cells after treatment with various concentrations of DIG

The RR *in situ* activity was measured in AsPC-1, BxPC-3, PANC-1 cells after incubation with various increasing concentrations of DIG. AsPC-1 was exposed to 20, 25, 30, and 35  $\mu\text{M}$  DIG. After 24 hours, the incorporation of  $^{14}\text{C}$ -cytidine was significantly reduced to 7%, 5%, 5%, and 4%. BxPC-3 and PANC-1 were exposed to 5, 10, 15 and 20  $\mu\text{M}$  DIG for 24 hours. The incorporation of  $^{14}\text{C}$ -cytidine into nascent DNA significantly decreased in both cancer cell lines (BxPC-3 to 2%, 0%, 1%, and 1% and PANC-1 to 3%, 0%, 0%, and 1%) (figures 18a-c).



Figures 18a-c: Inhibition of  $^{14}\text{C}$ -Incorporation into DNA of AsPC-1 (a), BxPC-3 (b), PANC-1 (c) cells after treatment with DIG for 24 hours.

#### 4.2.4. Cell cycle distribution in AsPC-1, BxPC-3, PANC-1 cells after treatment with DIG

Pancreatic cancer cell lines were incubated with different concentrations of DIG for 24 hours. Treatment of AsPC-1 cells with 30  $\mu\text{M}$  DIG caused an accumulation of cells in S phase,

increasing this cell population from 13% to 22%, whereas G2-M phase cells decreased from 32% to 16%. Growth arrest in BxPC-3 cells after exposure to 12.5 $\mu$ M DIG occurred mainly in the G2-M phase, increasing the cell population from 18% to 12.5% while depleting cells in the S-phase from 38% to 26%. In PANC-1 cells, a significant decrease or increase of cells in specific cell cycle phases was not observed at any concentration applied (figure 19a-c).

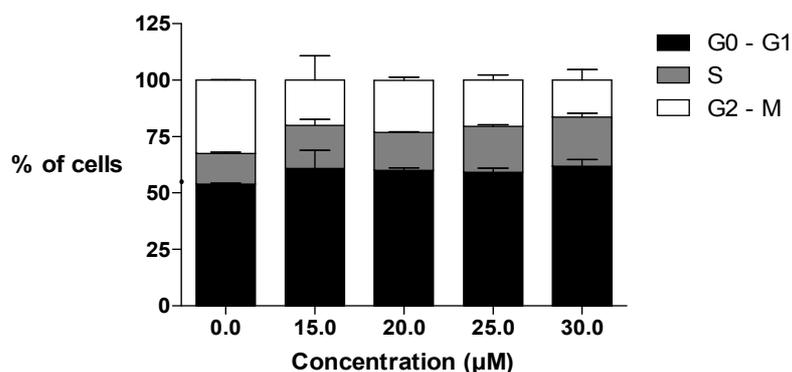


Figure 19a: AsPC-1 cell cycle distribution after incubation with DIG

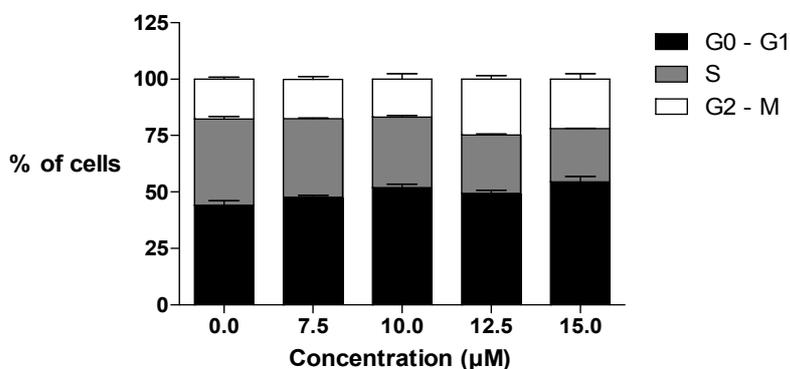


Figure 19b: BxPC-3 cell cycle distribution after incubation with DIG

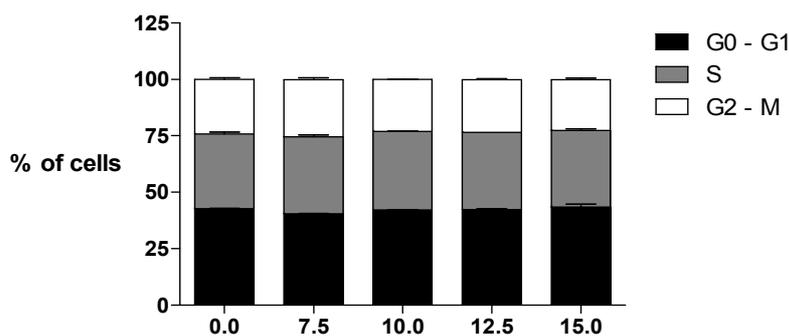


Figure 19c: PANC-1 cell cycle distribution after incubation with DIG

#### 4.2.5. Induction of apoptosis in AsPC-1, BxPC-3, PANC-1 cells by DIG

Pancreatic cancer cells were exposed to increasing concentrations of DIG for 72 hours and double stained with Hoechst 33258 and propidium iodide to analyze whether apoptotic cell death was induced. Neither AsPC-1 nor PANC-1 cells showed any significant apoptosis stages. 8.5% of AsPC-1 cells showed early as well as late apoptosis stages upon treatment with 30 $\mu$ M DIG (figure 20a); upon treatment with 7.5 and 15 $\mu$ M DIG approximately 6.5% of PANC-1 cells showed the same apoptotic and necrotic effects (figure 20b). For technical reasons apoptosis induction could not be evaluated in BxPC-3 cells. In line with these findings, western blot analysis after treatment of AsPC-1 cells with 40 $\mu$ M DIG showed that caspase 3 protein level remained unchanged.

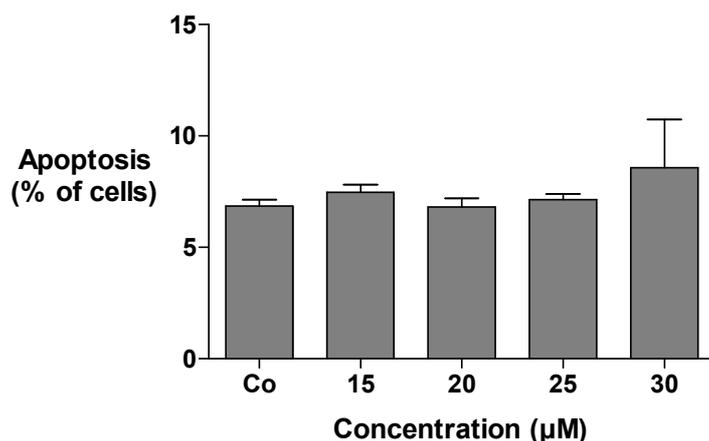


Figure 20a: Induction of apoptosis in AsPC-1 cells after treatment with DIG

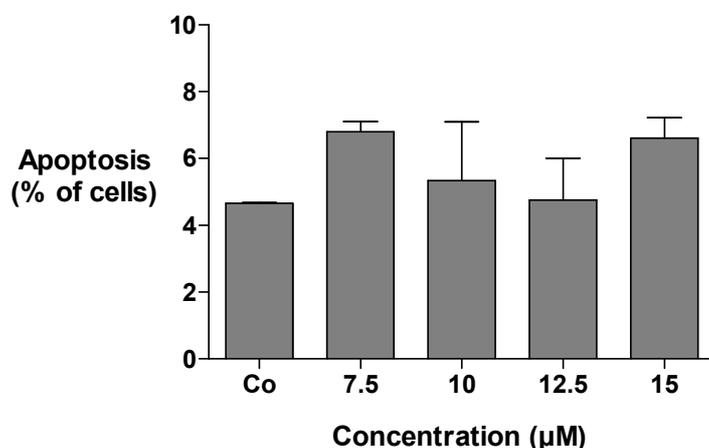
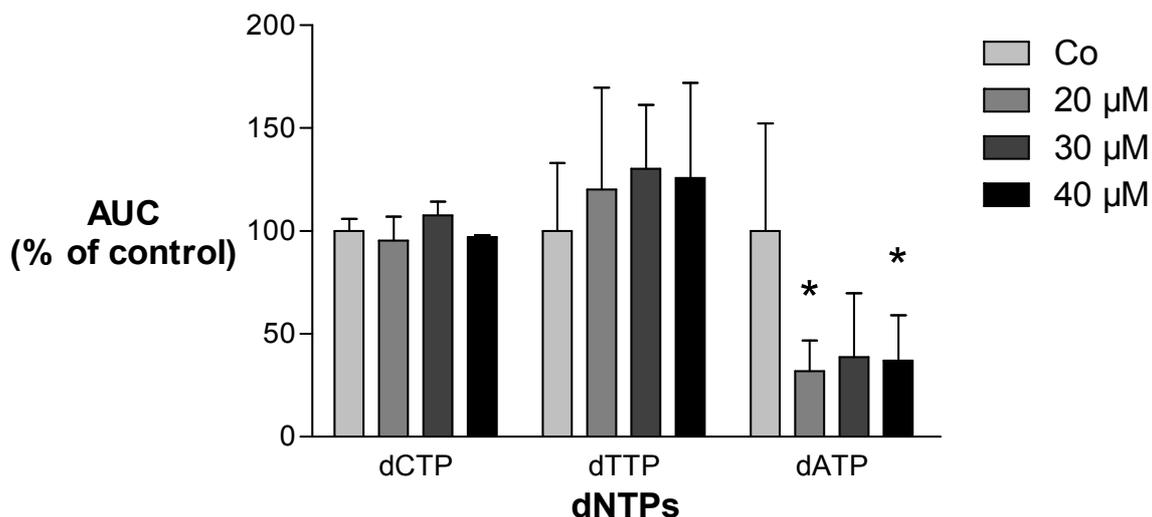


Figure 20b: Induction of apoptosis in PANC-1 cells after treatment with DIG

#### 4.2.6. Alterations of dNTP pool sizes in AsPC-1 cells after treatment with DIG

AsPC-1 cells were incubated with 20, 30, 40 $\mu$ M DIG for 24 hours. Then the dNTP pool sizes were determined using the HPLC method described in the methods section. Treatment with DIG caused an imbalance of dNTP pool sizes in AsPC-1 cells. A depletion of dATP concentrations to 31% of control values could be observed after treatment with DIG. While dCTP remained unaltered, dTTP concentrations increased up to 130% of control values after incubation of cells with DIG. All dGTP pools remained beyond the detectability of the method (figure 21).



**Figure 21: Concentration of dNTP pools in AsPC-1 cells after treatment with DIG for 24 hours**

#### 4.2.7. Expression of checkpoint and cell cycle regulating proteins after treatment of AsPC-1 cells with DIG

To investigate the expression of cell cycle regulators, AsPC-1 cells were treated with 40 $\mu$ M DIG for 0.5, 2, 4, 8, and 24 hours and examined by western blot analysis. Chk2 was only marginally phosphorylated at the activating Thr68 site within 24 hours. Chk2 levels remained unchanged. Furthermore, DIG caused phosphorylation at Ser177 of phosphatase Cdc25A, which is a target of Chk2, resulting in a transient induction of Cdc25A. In addition, treatment with DIG resulted in phosphorylation at Ser1981 of ATM kinase, which requires DNA damage and causes phosphorylation of Chk2 (figure 22).

### ASPC-1 + 40 $\mu$ M DIG

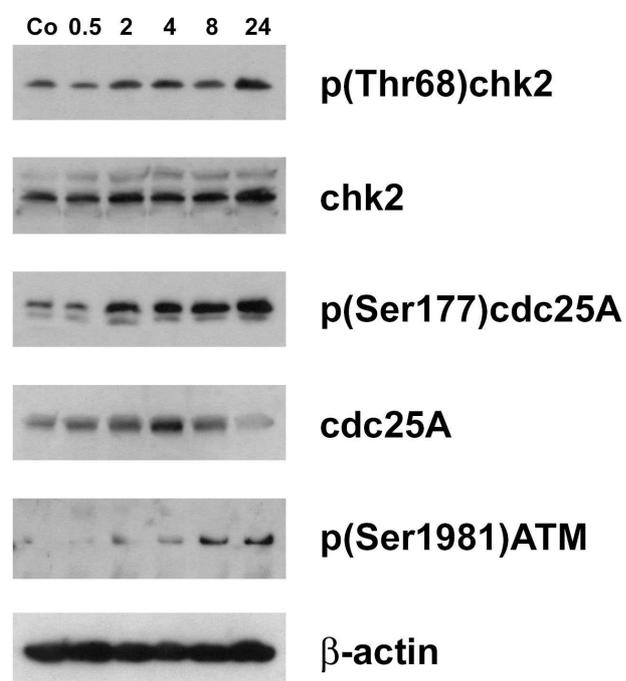


Figure 22: Expression of cell cycle regulators after treatment of AsPC-1 cells with 40 $\mu$ M DIG

#### 4.2.8. Expression of mitogen activated protein kinases after treatment of AsPC-1 with DIG

Western blot analysis demonstrated the effect of 40 $\mu$ M DIG on mitogen-activated proteins in AsPC-1 cells after treatment for 0.5, 2, 4, 8, and 24 hours. Phosphorylation of Erk1/2 was reduced within 4 hours. However, DIG showed a marginal induction effect on the p38 pathway. In addition, treatment with DIG resulted in phosphorylation at Ser473 of Akt kinase within 2 hours (figure 23).

### ASPC-1 + 40 $\mu$ M DIG

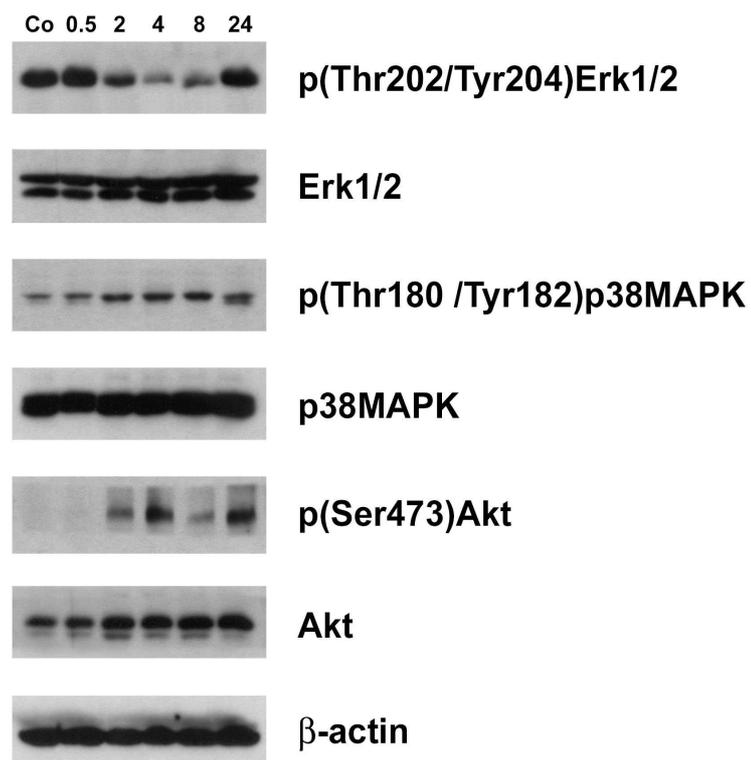


Figure 23: Western blot analysis demonstrating the effect of 40 $\mu$ M DIG in AsPC-1 cells on mitogen-activated protein kinase signaling pathways

## 5. Discussion

### 5.1. ABNM-13

A panel of 13 novel compounds (ABNM 1-13) was synthesized by a 3D molecular space modeling technique aiming at the inhibition of RR activity. The enzyme represents the rate-limiting factor of DNA synthesis. Growth inhibition assays in human HL-60 promyelocytic leukemia cells with all newly synthesized agents revealed that ABNM-13 was the most active compound.

Subsequently, ABNM-13 was shown to strongly inhibit the *in situ* RR activity despite low concentrations and a short incubation time. Furthermore, this newly designed agent caused alterations of the dNTP pools thereby causing a significant depletion of the dGTP pools while elevating the dTTP pools. Due to that imbalance *de novo* DNA synthesis is disturbed and cell proliferation blocked. In line with this, ABNM-13 treatment also caused cell cycle perturbations, induction of apoptosis, and growth arrest of HL-60 cells.

ABNM-13 led to a strong S-phase arrest, which is correlative with important RR properties: The enzyme forms the rate limiting factor for S-phase transit and inhibition of RR thus leads to inhibition of cells in S-phase (Chimploy et al, 2009).

The expression level of the R1 subunit protein of RR remained unchanged while the S-phase specific R2 and p53R2 subunits were elevated although HL-60 cells are normally p53 deficient. This finding suggests the assumption that cells try to rebalance the dNTP production by a compensatory up-regulation of p53R2 and is in line with recent observations made by Yanamoto et al showing that the basal level of p53R2 protein is expressed independent of the cellular p53 status (Yanamoto et al, 2005). Furthermore, Zhang et al demonstrated that up-regulation of R2 correlates with DNA damage and causes up-regulation and activation of Chk1 (Zhang et al, 2009).

Cell cycle checkpoint kinases are activated by DNA damage, imbalance of dNTP pools or by incomplete DNA replication. That difficult control system prevents DNA synthesis, interrupts further cell cycle progression and provides time for either reconstitution of dNTP balance or DNA repair before the damage gets passed on to daughter cells (Kastan and Bartek, 2004; Shiloh, 2003; Bartek and Lukas, 2003).

Before replicating cells undergo mitosis, three major cell cycle checkpoint kinases have to be passed. Up-regulation and activation of these checkpoint proteins is controlled by specific key regulator proteins, ATR and ATM kinases. ATR activates downstream effector kinase Chk1 while ATM activates effector kinase Chk2 by phosphorylation (Bartek and Lukas, 2003; Abraham, 2001). Activation of Chk1 or Chk2 results in phosphorylation of Cdc25A phosphatase at Ser75 and Ser177, respectively. Phosphorylation of Cdc25A oncogene tags the protein for proteasomal degradation. The phosphatase is required for maintaining cell cycle transit. Treatment with 15 $\mu$ M ABNM-13 activated both effector kinases Chk1 and Chk2, respectively. Chk2 protein was phosphorylated within 30 min.

Phosphatases Cdc25B and Cdc25C induce cells entering mitosis by activating Cdk1/ cyclin B (Donzelli and Draetta, 2003), whereas Cdc25B is indicated to be the initial phosphatase to activate Cdk1/ cyclin B protein (Nishijima et al, 1997). Activation causes phosphorylation and activation of Cdc25C, which keeps Cdk1/cyclin B active. That mechanism represents a positive feedback loop, which escorts replicating cells through mitosis (Hoffmann et al, 1993).

Treatment with ABNM-13 led to up-regulation of Cdc25B levels which results in dephosphorylation and activation of Cdk1. The expression level of Cdc25C remained unchanged. However, Ara-C caused induction of Cdk1/cyclin B protein expression; simultaneous co-treatment with Ara-C and ABNM-13 induced an increase of Cdk1 expression level as well as increase of its activity. These observations showed that cell cycle arrest is induced by preventing entry into mitosis (Varmeh-Ziaie and Manfredi, 2007).

Cdk2 protein level is regulated by Cdc25A and is needed for S-phase progression (Donzelli et al, 2002). Hence, the combination of Cdc25B overexpression and Cdc25A degradation resulted in a nearly complete S-phase arrest by treatment with 15 $\mu$ M ABNM-13 alone or in combination with 15nM Ara-C.

ABNM-13 causes apoptosis in only 22% of all HL-60 cells (after 48 hours). This observation supports the conclusion that cell cycle inhibition rather than induction of apoptosis is the primary antineoplastic effect of this novel agent.

We therefore believe that a portion of treated cells was growing much slower than untreated controls, but did not undergo necrosis or apoptosis. Apoptosis was determined by the level of cleaved caspase 3 protein expression after 8 hours. Elevation of cleaved caspase 3 led to increase of  $\gamma$ H2AX protein expression level after incubation with ABNM-13 for 24 hours.

These observations led us to the conclusion that treatment with ABNM-13 did not primarily cause DNA double strand breaks but rather was the result of caspase-3 induced DNase activation. Supporting that theory, constitutive phosphor-ATM protein level remained unchanged.

Cell death caused by mitotic catastrophe (i.e. the formation of giant cells with more than one nucleus) being induced by inhibition of Chk2 protein expression (Portugal et al, 2010; Castedo et al, 2004) could not be observed at any time point.

Finally, ABNM-13 was combined with Ara-C, a well established first-line antitumor agent. Combination treatment is expected to produce fortified antitumor effects, if the pharmacokinetic and pharmacological properties are different from each other. Accordingly, ABNM-13, which disregulated dTTP and dGTP pools and Ara-C, which is known to affect dCTP pools (Gandhi et al, 1997; Wills et al, 2000; Seymour et al, 1996) inhibited cell proliferation synergistically. Employing a sequential growth inhibition assay with ABNM-13 and Ara-C, all 12 concentrations applied resulted in highly synergistic antineoplastic effects.

ABNM-13 exerts pronounced anticancer activity as single drug as well as in combination with Ara-C. In addition, it has been shown that ABNM-13 acts as an effective RR inhibitor. Due to these promising results, we believe that further investigations and *in vivo* studies with this novel antitumor drug are warranted and we therefore consider ABNM-13 as a potent candidate for effective future cancer treatment options.

## 5.2. DIG

Pancreatic cancer is a very aggressive, malignant neoplasm with poor prognosis and short survival rate. Digalloylresveratrol (DIG) is a novel synthetic ester of the natural compounds gallic acid and resveratrol and was suggested to exhibit promising antitumor activity in pancreatic cancer cells to possibly support conventional chemotherapy in the near future.

Bernhaus et al demonstrated that growth inhibition of HT-29 colon cancer cells after treatment with DIG was superior to incubation with an equimolar combination of RV and GA (Bernhaus et al, 2009). In HL-60 cells the inhibition of cell proliferation of DIG also exceeded that of GA by 10-fold (Madlener et al, 2007). These results support the conclusion that the RV backbones, to which galloyl-residues are connected, are responsible for the determined additive effects (Madlener et al, 2010).

Interestingly, clonogenic assays with DIG using three different pancreatic cancer cell lines (AsPC-1, BxPC-3, PANC-1) did not show any similar effects. Different cellular morphology and pharmacology might be an explanation for these surprising results. Furthermore, DIG was combined with dFdC, a well established first-line chemotherapeutic drug against pancreatic cancer. At the concentrations applied, no synergistic effects could be observed in AsPC-1 cells. These results support the conclusion that DIG and dFdC do not affect cell proliferation in a different way as it was revealed with ABNM-13 and Ara-C.

Recent investigations showed that DIG inhibits RR by chelating the tyrosyl radical being essential for RR activity (Madlener et al, 2007). The *in vitro* radical-scavenging activity of DIG exceeded the equimolar combination of RV and GA by about 2.5-fold. These results are in correlation with the measurement of RR *in situ* activity by <sup>14</sup>C-cytidine assay. We could demonstrate that DIG significantly inhibited RR *in situ* activity in all pancreatic cell lines.

In addition, DIG led to alterations of dNTP pool balance in AsPC-1 cells: dATP pools were significantly depleted while dCTP pools remained unchanged, and dTTP pools were marginally elevated. That imbalance of dNTP pools finally blocks DNA synthesis and subsequent cell proliferation. Perturbation of dNTP pools and inhibition of RR *in situ* activity are the consequences of treatment with DIG. These results are consistent with observations concerning the cell cycle regulation. DIG caused an S-phase arrest in AsPC-1 cells, which

confirms the fact that RR inhibition results in blocking the S-phase transit of proliferating cells (Chimpoy et al, 2009).

In contrast, treatment with DIG caused G2-M arrest in BxPC-3 cells while PANC-1 cells did not show any effect.

It was demonstrated that DIG exhibits strong pro-apoptotic properties in HL-60 cells and triggers apoptosis by the caspase 3 pathway (Madlener et al, 2010). Apoptosis in pancreatic cell lines (AsPC-1 and PANC-1) upon treatment with DIG occurred only in a negligible rate of cells after 72 hours and accordingly, the expression level of caspase 3 protein in AsPC-1 cells remained unchanged. These results indicate that DIG as well as ABNM-13 is rather an inhibitor of the cell cycle than an inducer of programmed cell death.

Because of cell perturbations, checkpoint proteins are activated preventing DNA synthesis and halting cell cycle progression (Kastan and Bartek, 2004; Shiloh, 2003; Bartek and Lukas, 2003). Cell cycle checkpoint effector kinases Chk1 and Chk2 are activated by ATR or ATM, respectively. It is believed that these protein kinases are activated in the presence of broken DNA double strands. Treatment with DIG in AsPC-1 cells shows an up-regulation/phosphorylation of ATM, which indicates DNA double strand breaks. These observations indicate that ATM up-regulation is important in response of DNA alkylation and not limited to ionizing radiation-induced response (Ke-Qing and Suo-Jiang, 2003).

DIG treatment led to activation of Chk2 in AsPC-1 cells, which was paralleled with phosphorylation of Cdc25A phosphatase at Ser177 and targeted it for protein degradation after 8 hours. Cdc25A is an oncogene and essential for cell cycle transit.

Using AsPC-1 cells, western blot analysis of p38MAPK, Erk1/2, and Akt mitogen-activated protein kinases (MAPK) showed that DIG blocked Erk1/2 phosphorylation in the cytoplasm while inducing phosphorylation of p38 and Akt kinases. MAPK enzymes are important connections between cell surface receptors and critical regulatory targets. These enzymes answer to physical and chemical stress (e.g. chemotherapeutic substances) by regulating most cellular processes, from specific protein expression to programmed cell death (Johnson and Lapadat, 2002).

Fenton et al also observed an inhibition of the Erk1/2 cytoplasmic pathway in a specific papillary thyroid cancer cell line after treatment with Sunitinib, an inhibitor of receptor tyrosine kinases. It is suggested that blocking this pathway is part of a mechanism, which

results in inhibition of cell proliferation and causes up-regulation of *NIS* gene expression. Fenton and co-workers demonstrated that Sunitinib induces expression of sodium (Na)/iodide (I) symporters (NIS) (Fenton et al, 2010).

Inhibition of Erk1/2 pathway in AsPC-1 after treatment with DIG may induce expression of a similar protein resulting in more effective cell regulation and homeostasis.

DIG exhibits significant inhibition of the *in situ* RR activity, induction of cell cycle arrest, and a high *in vitro* radical-scavenging activity. Taken together, it could be demonstrated that DIG exerts promising anticancer effects in pancreatic cancer cells and may be of interest to preclinical investigations and *in vivo* studies.

## 6. Abstract

The enzyme ribonucleotide reductase (RR) is essential for the conversion of ribonucleoside diphosphates into deoxyribonucleoside diphosphates, which are the basic units for DNA synthesis and DNA repair. *De novo* dNTP synthesis is an exactly regulated reaction, thus making RR the rate-limiting enzyme. Because of its up-regulation in malignant tumor cells, RR is considered to be a sensitive target for cytotoxic chemotherapeutic agents.

In this diploma thesis, two different novel compounds were investigated for their anticancer activity in various human tumor cell lines. ABNM-13 is a novel designed N-hydroxy-N'-aminoguanidine using 3D molecular space modeling techniques while Digalloylresveratrol (DIG) represents a novel synthetic ester of the polyhydroxy phenolic substances resveratrol (3,4,5-trihydroxystilbene; RV) and gallic acid (3,4,5-trihydroxybenzoic acid; GA). Both components of DIG are naturally occurring substances with promising results in cancer treatment. Biochemical effects of ABNM-13 were investigated in human HL-60 promyelocytic leukemia cells whereas effects of DIG were evaluated in three different pancreatic cancer cell lines (AsPC-1, BxPC-3, and PANC-1). ABNM-13 as well as DIG were suggested to inhibit RR activity causing block of DNA replication, cell perturbations, induction of programmed cell death, and growth arrest.

Indeed, both novel agents proved to be effective inhibitors of RR by significantly lowering the incorporation of  $^{14}\text{C}$ -labeled cytidine into DNA of tumor cells. Furthermore, both compounds caused alterations of dNTP pool balance and provoked cell cycle arrest and activation of cell cycle checkpoint effector kinases Chk1 and Chk2. Inhibition of RR activity results in activation of Chk1 and/or Chk2, leading to degradation of Cdc25A, which is responsible for cell cycle transit.

ABNM-13 treatment of HL-60 cells further led to up-regulation of Cdc25B, which caused dephosphorylation and activation of Chk1. Dysregulation of both Cdc25A and Cdc25B induced cell cycle arrest in S-phase.

Western blot analysis after treatment of AsPC-1 cells with DIG demonstrated that inhibition of RR activity causes replicative stress, which results in up-regulation of mitogen-activated

protein kinases p38 and Akt. Interestingly, an inhibition of Erk1/2 cytoplasmic pathway could also be observed.

Our investigations also showed that the primary antineoplastic effect of DIG as well as ABNM-13 is rather inhibition of the cell cycle than induction of programmed cell death.

ABNM-13 was then combined with Cytarabine (Ara-C), a first-line cytotoxic agent resulting in a synergistic potentiation of the antineoplastic effect of Ara-C. In contrast, DIG in combination with Gemcitabine (dFdC) did not show any synergistic effects.

Due to these promising results, ABNM-13 and DIG may be potential candidates for further preclinical investigations and *in vivo* studies.

## 7. Zusammenfassung

Ribonukleotid Reduktase (RR) ist ein Enzym, das für die Konvertierung von Ribonukleotiddiphosphaten in Desoxyribonukleotiddiphosphate essentiell ist. Diese Bausteine bilden die Grundlage für DNA Synthese und DNA Reparatur. Die *de novo* dNTP Synthese stellt eine exakt regulierte Reaktion dar, weshalb RR hier als limitierendes Enzym fungiert. Zahlreiche Untersuchungen ergaben, dass die Expression von RR in Tumorzellen hochreguliert wird, wodurch dieses Enzym für zytotoxische chemotherapeutische Substanzen ein sensibles Ziel darstellt.

Im Zuge dieser Diplomarbeit wurden zwei neue Substanzen untersucht, von welchen man sich in verschiedenen humanen Tumorzelllinien antikanzerogene Wirkung erhoffte. ABNM-13 ist ein neu synthetisiertes N-hydroxy-N'-aminoguanidin welches mittels 3D molecular space modeling Technikenentworfen wurde, um gezielt die RR Aktivität zu hemmen. Digalloylresveratrol (DIG) hingegen ist ein synthetisch hergestellter Ester, zusammengesetzt aus den natürlich vorkommenden polyhydroxyphenolischen Substanzen Resveratrol (3,4,5-trihydroxystilbene; RV) und Gallussäure (3,4,5-trihydroxybenzoic acid; GA). Jede der beiden Einzelkomponenten zeigte bereits in früheren Untersuchungen vielversprechende Ergebnisse in der Krebsbehandlung.

Die biochemische Wirkung von ABNM-13 wurde in humanen HL-60 Promyelozyten-Leukämiezellen untersucht, wohingegen die Effekte von DIG in verschiedenen Pankreaskarzinomzelllinien (AsPC-1, BxPC-3, PANC-1) nachgewiesen werden sollten. Durch einen möglichen inhibierenden Effekt beider Substanzen auf die Aktivität der RR wäre eine Störung des Zellstoffwechsels die Folge, die in einer Hemmung der DNA Replikation, einem Arrest des Zellwachstums und möglicherweise Induktion von Apoptose resultiert.

Sowohl ABNM-13 als auch DIG bewirkten aufgrund erster Untersuchungen tatsächlich eine signifikante Hemmung der RR Aktivität, was durch die Abnahme des Einbaus von <sup>14</sup>C-markiertem Cytidin in die DNA von Tumorzellen gezeigt werden konnte. Des Weiteren konnte demonstriert werden, dass die ausgewogene Verteilung der dNTP Pools durch die Behandlung mit ABNM-13 und DIG verändert wird. Beide Substanzen greifen in die Regulation des Zellzyklus ein, was schließlich zur Aktivierung der spezifischen Checkpoint Effektorinasen Chk1 und Chk2 führt. Durch die Hemmung der RR Aktivität wird Chk1

und/oder Chk2 aktiviert, was wiederum den Abbau von Cdc25A bewirkt. Cdc25A ist ein Onkogen, das beim Zellzyklus-Transit von entscheidender Bedeutung ist.

Die Behandlung von HL-60 Zellen mit ABNM-13 bewirkt zusätzlich eine Erhöhung des Cdc25B Proteinlevels, was Dephosphorylierung und Aktivierung von Chk1 zur Folge hat. Durch diese Dysregulation von Cdc25A und Cdc25B kann in HL-60 Zellen in weiterer Folge ein S-Phase Arrest beobachtet werden.

Western Blot Analysen nach Behandlung in AsPC-1 Zellen mit DIG zeigten, dass eine Hemmung der RR Aktivität die Erhöhung des Expressionslevels von mitogen-aktiverenden Proteinkinasen p38 und Akt bewirkt. Im Gegensatz dazu wurde allerdings auch eine Hemmung des zytoplasmatischen Erk1/2 Signalweges beobachtet.

Interessanterweise induzieren beide Substanzen nur in sehr geringem Maße Apoptose. Das legt den Schluss nahe, dass sowohl ABNM-13 als auch DIG eher hemmend auf den Zellzyklus als induzierend auf den programmierten Zelltod wirken.

Schließlich wurde gezeigt, dass ABNM-13 in Kombination mit Cytarabine (Ara-C), einem bewährten „first-line“ Zytostatikum, zu einer signifikanten Verstärkung des antineoplastischen Effekts von Ara-C führt. Im Gegensatz dazu konnte DIG in Kombination mit Gemcitabine (dFdC) keinen ähnlichen Effekt erzielen.

Aufgrund dieser vielversprechenden Ergebnisse und Erkenntnisse könnten ABNM-13 und DIG zu potentiellen Anwärtern für etwaige präklinische Untersuchungen oder *in vivo* Studien werden.

## 8. References

- Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Development* 2001;15:2177–96.
- Ahlgren JD. Epidemiology and risk factors in pancreatic cancer. *Semin Oncol* 1996;23:241–50.
- Bakkevold KE, Arnesjo B, Dahl O, Kambestad B. Adjuvant combination chemotherapy (AMF) following radical resection of carcinoma of the pancreas and papilla of Vater – results of a controlled, prospective, randomised multicentre study. *Eur J Cancer* 1993; 29A: 698–703.
- Banu E, Banu A, Fodor A, et al: Meta-analysis of randomized trials comparing gemcitabine-based doublets versus gemcitabine alone in patients with advanced and metastatic pancreatic cancer. *Drugs Asing* 24: 865-879, 2007.
- Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. *Environ Health Perspect.* 1993 Apr;100:9-20. Review. PMID: 8354184
- Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 2003;3:421–9.
- Baur JA, Sinclair DA Therapeutic potential of resveratrol: the in vivo evidence *Nat Rev Drug Discov.* 2006 Jun;5(6):493-506. Epub 2006 May 26. Review.PMID: 16732220
- Ben-David Y, Bernstein A. Friend virus-induced erythroleukemia and the multistage nature of cancer. *Cell.* 1991;66: 831–834.
- Bernhaus A, Fritzer-Szekeres M, Grusch M, Saiko P, Krupitza G, Venkateswarlu S, Trimurtulu G, Jaeger W, Szekeres T. Digalloyl-resveratrol, a new phenolic acid derivative induces apoptosis and cell cycle arrest in human HT-29 colon cancer cells. *Cancer Lett.* 2009;274(2):299–304.
- Bittencourt AL, Primo J, Oliveira MF Manifestations of the human T-cell lymphotropic virus type I infection in childhood and adolescence. *J Pediatr (Rio J).* 2006 Nov-Dec;82(6):411-20. Review.PMID: 17171202
- Boyle P, Hsieh CC, Maisonneuve P, La Vecchia C, Macfarlane GJ, Walker AM, Trichopoulos D. Epidemiology of pancreas cancer (1988). *Int J Pancreatol.* 1989 Dec;5(4):327-46. Review.PMID: 2691590
- Burriss HA, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; 15: 2403–13.

- Carmichael J, Fink U, Russell R, et al. Phase II study of gemcitabine in patients with advanced pancreatic cancer. *Br J Cancer* 1996; 73: 101–5.
- Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 2004;23:2825–37.
- Chimply K, Diaz GD, Li Q, Carter O, Dashwood WM, Mathews CK, et al. E2F4 and ribonucleotide reductase mediate S-phase arrest in colon cancer cells treated with chlorophyllin. *International Journal of Cancer* 2009;125:2086–94.
- Chou TC, Talalay P. Generalized equations for the analysis of inhibitions of Michaelis–Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. *European Journal of Biochemistry (FEBS)* 1981;115:207–16.
- Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Advances in Enzyme Regulation* 1984;22:27–55
- Cooperman, B. S.; Kashlan, O. B. A comprehensive model for the allosteric regulation of Class Ia ribonucleotide reductases. *Adv. Enzyme Regul.* 2003, 43, 167-182.
- Cui J, Sun R, Yu Y, Gou S, Zhao G, Wang C. Antiproliferative effect of resveratrol in pancreatic cancer cells. *Phyther Res.* 2010 Nov;24(11):1637-44. doi: 10.1002/ptr.3157.PMID: 21031621
- Cullinan S, Moertel CG, Wieand HS, et al. A phase III trial on the therapy of advanced pancreatic carcinoma. Evaluations of the Mallinson regimen and combined 5-fluorouracil, doxorubicin, and cisplatin. *Cancer* 1990; 65: 2207–12.
- De Beer D, Joubert E, Gelderblom WC, Manley M. Antioxidant activity of South African red and white cultivar wines: free radical scavenging. *J Agric Food Chem* 2003;51:902–909. [PubMed: 12568547]
- Deschler B, Lübbert M. Acute myeloid leukemia: epidemiology and etiology. *Cancer.* 2006 Nov 1;107(9):2099-107. Review.PMID: 17019734
- Di Marco M, Di Cicilia R, Macchini M, Nobili E, Vecchiarelli S, Brandi G, Biasco G. Metastatic pancreatic cancer: is gemcitabine still the best standard treatment? (Review). *Oncol Rep.* 2010 May;23(5):1183-92. PMID: 20372829
- Doll R, Peto R, Wheatley K et al. Mortality in relation to smoking: 40 years' observations on male British doctors. *Br Med J* 1994; 309(6959): 901–911
- Donzelli M, Draetta GF. Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Reports* 2003;4:671–7.
- Donzelli M, Squatrito M, Ganoth D, Hershko A, Pagano M, Draetta GF. Dual mode of degradation of Cdc25 A phosphatase. *EMBO Journal* 2002;21:4875–84.

- Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. Major cell death pathways at a glance *Microbes Infect.* 2009 Nov;11(13):1050-62. Epub 2009 Sep 4. Review.PMID: 19733681
- Eichhorst BF, Busch R, Hopfinger G, Pasold R, Hensel M, Steinbrecher C, Siehl S, Jäger U, Bergmann M, Stilgenbauer S, Schweighofer C, Wendtner CM, Döhner H, Brittinger G, Emmerich B, Hallek M Fludarabine plus cyclophosphamide versus fludarabine alone in first-line therapy of younger patients with chronic lymphocytic leukemia. *Blood.* 2006 Feb 1;107(3):885-91. Epub 2005 Oct 11. PMID: 16219797
- Faderl, S.; Gandhi, V.; Kantarjian, H.; Plunkett, W. New nucleoside analogues in clinical development. *Cancer Chemother. Biol. Response Modif.* 2002, 20, 37-58.
- Falck J, Petrini JH, Williams BR, Lukas J, Bartek J. The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways *Nat Genet.* 2002 Mar;30(3):290-4. Epub 2002 Feb 19.PMID: 11850621
- Fenton MS, Marion KM, Salem AK, Hogen R, Naeim F, Hershman JM. Sunitinib inhibits MEK/ERK and SAPK/JNK pathways and increases sodium/iodide symporter expression in papillary thyroid cancer. *Thyroid.* 2010 Sep;20(9):965-74.PMID: 20629553
- Forman D, Stockton D, Moller H, et al. Cancer prevalence in the UK: results from the EUROPREVAL study. *Ann Oncol.* 2003;14:648–654.
- Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J.* 2004 Dec 1;384(Pt 2):201-32. PMID: 15450003
- Gagner M, Pomp A Laparoscopic pylorus-preserving pancreatoduodenectomy. *Surg Endosc.* 1994 May;8(5):408-10.PMID: 7915434
- Gagner M, Pomp A. Laparoscopic pancreatic resection: Is it worthwhile? *J Gastrointest Surg.* 1997 Jan-Feb;1(1):20-5; discussion 25-6.PMID: 9834326
- Gandhi V, Huang P, Chapman AJ, Chen F, Plunkett W. Incorporation of fludarabine and 1-beta-D-arabinofuranosylcytosine 50-triphosphates by DNA polymerase alpha: affinity, interaction, and consequences. *Clinical Cancer Research* 1997;3:1347–55.
- Gelibter A, Malaguti P, Di Cosimo S, et al: Fixed dose-rate gemcitabine infusion as first-line treatment for advanced-stage carcinoma of the pancreas and biliary tree. *Cancer (Suppl)* 104: 1237-1245, 2005.
- Ghadirian P, Lynch HT, Krewski D. Epidemiology of pancreatic cancer: an overview. *Cancer Detect Prev.* 2003;27(2):87-93. PMID: 12670518
- Glimelius B, Hoffman K, Sjöden PO, Jacobsson G, Sellström H, Enander LK, Linne T, Svensson C. Chemotherapy improves survival and quality of life in advanced pancreatic and biliary cancer. *Ann Oncol.* 7: 593-600, 1996

- Gribben JG. Role of allogeneic hematopoietic stem-cell transplantation in chronic lymphocytic leukemia *J Clin Oncol*. 2008 Oct 20;26(30):4864-5. Epub 2008 Sep 15. No abstract available. PMID: 18794537
- Hanahan D, Weinberg A. The Hallmarks of Cancer. *Cell* 2000; 100:57-70. Review
- Hennings H, Glick AB, Greenhalgh DA, Morgan DL, Strickland JE, Tennenbaum T, Yuspa SH. Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis. *Proc Soc Exp Biol Med*. 1993 Jan;202(1):1-8. Review. PMID: 8424089
- Hoffmann I, Clarke PR, Marcote MJ, Karsenti E, Draetta G. Phosphorylation and activation of human cdc25-C by cdc2—cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO Journal* 1993;12:53–63.
- Horvath Z, Murias M, Saiko P, Erker T, Handler N, Madlener S, Jaeger W, Grusch M, Fritzer-Szekeres M, Krupitza G, Szekeres T. Cytotoxic and biochemical effects of 3,3',4,4',5,5'-hexahydroxystilbene, a novel resveratrol analog in HL-60 human promyelocytic leukemia cells. *Exp Hematol*. 2006;34(10):1377–1384.
- Horvath Z, Saiko P, Illmer C, Madlener S, Hoechtl T, Bauer W, Erker T, Jaeger W, Fritzer-Szekeres M, Szekeres T. Synergistic action of resveratrol, an ingredient of wine, with Ara-C and tiazofurin in HL-60 human promyelocytic leukemia cells. *Exp Hematol* 2005;33(3):329–335. [PubMed: 15730856]
- Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2 $\beta$ ,2 $\beta$ -difluorodeoxycytidine on DNA synthesis. *Cancer Res* 1991; 51: 6110–7.
- Huxley R, Ansary-Moghaddam A, Berrington dG et al. Type-II diabetes and pancreatic cancer: a metaanalysis of 36 studies. *Br J Cancer* 2005; 92(11): 2076–2083.
- Isuzugawa K, Inoue M, Ogihara Y. Catalase contents in cells determine sensitivity to the apoptosis inducer gallic acid, *Biol. Pharm Bull* 2001;24:1022–1026.
- Jesnowski R, Isaksson B, Möhrcke C, Bertsch C, Bulajic M, Schneider-Brachert W, Klöppel G, Lowenfels AB, Maisonneuve P, Löhr JM. *Helicobacter pylori* in autoimmune pancreatitis and pancreatic carcinoma. *Pancreatology*. 2010;10(4):462-6. Epub 2010 Aug 19. PMID: 20720447
- Johannsson O, Ostermeyer EA, Hakansson S, et al. Founding BRCA1 mutations in hereditary breast and ovarian cancer in southern Sweden. *Am J Hum Genet* 1996;58:441–50.
- Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002 Dec 6;298(5600):1911-2. Review. PMID: 12471242
- Kalser MH, Ellenberg SS. Pancreatic cancer. Adjuvant combined radiation and chemotherapy following curative resection. *Arch Surg* 1985; 120: 899–903.

- Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004 Nov 18;432(7015):316-23. Review.PMID: 15549093
- Kawada M, Ohno Y, Ri Y, Ikoma T, Yuugetu H, Asai T, Watanabe M, Yasuda N, Akao S, Takemura G, Minatoguchi S, Gotoh K, Fujiwara H, Fukuda K. Anti-tumor effects of gallic acid on LL-2 lung cancer cells transplanted in mice. *Anticancer Drugs* 2001;12:847–852. [PubMed: 11707653]
- Keating MJ, O'Brien S, Albitar M, Lerner S, Plunkett W, Giles F, Andreeff M, Cortes J, Faderl S, Thomas D, Koller C, Wierda W, Detry MA, Lynn A, Kantarjian H. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. *J Clin Oncol*. 2005 Jun 20;23(18):4079-88. Epub 2005 Mar 14.PMID: 15767648
- Kolberg, M.; Strand, K. R.; Graff, P.; Andersson, K. K. Structure, function, and mechanism of ribonucleotide reductases. *Biochim. Biophys. Acta* 2004, 1699, 1-34.
- Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods*. 2008 Mar;44(3):205-21. Review.PMID: 18314051
- Lassmann G, Thelander L, Gräslund A. EPR stopped-flow studies of the reaction of the tyrosyl radical of protein R2 from ribonucleotide reductase with hydroxyurea. *Biochem. Biophys. Res. Commun.* 1992, 188, 879-887
- Le Beau MM, Albain KS, Larson RA, et al. Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia: further evidence for characteristic abnormalities of chromosomes no. 5 and 7. *J Clin Oncol*. 1986;4:325–345.
- Lichtenstein P, Holm NV, Verkasalo PK et al. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med* 2000; 343(2):78–85.
- Lin Y, Tamakoshi A, Kawamura T et al. A prospective cohort study of cigarette smoking and pancreatic cancer in Japan. *Cancer Causes Control* 2002; 13(3): 249–254.
- Lin, Z. P.; Belcourt, M. F.; Cory, J. G.; Sartorelli, A. C. Stable suppression of the R2 subunit of ribonucleotide reductase by R2- targeted siRNA sensitizes p53 (-/-) HCT-116 colon cancer cells to DNA-damaging agents and ribonucleotide reductase inhibitors. *J. Biol. Chem.* 2004, 279, 27030-27038
- Lodish H, Berk A, Kaiser C, Krieger M, Scott M, Bretscher A, Ploegh H, Matsudaira P. *Molecular cell biology* (2008)
- Loos M, Kleeff J, Friess H and Buchler MW: Surgical treatment of pancreatic cancer. *Ann N Y Acad Sci* 1138: 169-180, 2008.
- Lowenfels AB, Maisonneuve P & Lankisch PG. Chronic pancreatitis and other risk factors for pancreatic cancer. *Gastroenterol Clin North Am* 1999; 28(3): 673–685

- Lowenfels AB, Maisonneuve P. Epidemiology and risk factors for pancreatic cancer. *Best Pract Res Clin Gastroenterol.* 2006 Apr;20(2):197-209. PMID: 16549324
- Lozano, G.; Elledge, S. J. p53 sends nucleotides to repair DNA. *Nature* 2000, 404, 24-25.
- Madlener S, Illmer C, Horvath Z, Saiko P, Losert A, Herbacek I, Grusch M, Elford HL, Krupitza G, Bernhaus A, Fritzer-Szekeres M, Szekeres T. Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukemia cells. *Cancer Lett.* 2007;245(1 – 2):156–162.
- Madlener S, Saiko P, Vonach C, Viola K, Huttary N, Stark N, Popescu R, Gridling M, Vo NT, Herbacek I, Davidovits A, Giessrigl B, Venkateswarlu S, Geleff S, Jäger W, Grusch M, Kerjaschki D, Mikulits W, Golakoti T, Fritzer-Szekeres M, Szekeres T, Krupitza G. Multifactorial anticancer effects of digalloyl-resveratrol encompass apoptosis, cell-cycle arrest, and inhibition of lymphendothelial gap formation in vitro. *Mutat Res.* 2010 Jan 5;683(1-2):123-30. Epub .PMID: 19909759
- Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol.* 2007 Sep;8(9):741-52. Review.PMID: 17717517
- Malet-Martino M, Martino R. Clinical studies of three oral prodrugs of 5-fluorouracil (capecitabine, UFT, S-1): a Review. *Oncologist* 2002; 7: 288–323.
- Meeran SM, Katiyar SK. Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Front Biosci.* 2008 Jan 1;13:2191-202. Review.PMID: 17981702
- Moertel CG, Frytak S, Hahn RG, O'Connell MJ, Reitemeier RJ, Rubin J, Schutt AJ, Weiland LH, Childs DS, Holbrook MA, Lavin PT, Livstone E, Spiro H, Knowlton A, Kalsner M, Barkin J, Lessner H, Mann-Kaplan R, Ramming K, Douglas HO Jr, Thomas P, Nave H, Bateman J, Lokich J, Brooks J, Chaffey J, Corson JM, Zamcheck N, Novak JW. Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil: The Gastrointestinal Tumor Study Group. *Cancer.* 1981 Oct 15;48(8):1705-10. PMID: 7284971
- Morgan DO. Principles of CDK regulation. *Nature.* 1995 Mar 9;374(6518):131-4. Review.PMID: 7877684
- Neoptolemos J, Cunningham D, Freiss H, et al. Adjuvant therapy in pancreatic cancer: historical and current perspectives. *Ann Oncol* 2003; 14: 675–92.
- Neuget AL, Habibul A, Robinson E. Pancreas cancer as a second primary malignancy: a population-based study. *Cancer* 1995;16:589–92.
- Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J Nat Prod.* 2007 Mar;70(3):461-77. Epub 2007 Feb 20. Review. PMID: 17309302.

- Niederhuber JE, Brennan MF, Menck HR. The National Cancer Data Base report on pancreatic cancer. *Cancer*. 1995 Nov 1;76(9):1671-7. PMID: 8635074
- Nishijima H, Nishitani H, Seki T, Nishimoto T. A dual-specificity phosphatase Cdc25B is an unstable protein and triggers p34(cdc2)/cyclin B activation in hamster BHK21 cells arrested with hydroxyurea. *Journal of Cell Biology* 1997;138:1105–16.
- Nocentini, G. Ribonucleotide reductase inhibitors: new strategies for cancer chemotherapy. *Crit. Rev. Oncol. Hematol.* 1996, 22, 89-126.
- Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. Toward maintaining the genome: DNA damage and replication checkpoints. *Annu Rev Genet.* 2002;36:617-56. Epub 2002 Jun 11. Review. PMID: 12429704
- Nordlund P., Reichard P., Ribonucleotide reductases, *Annu. Rev. Biochem.* 75 (2006) 681–706.
- Phelan CM, Lancaster JM, Tonin P, et al. Mutation analysis of the BRCA2 gene in 49 site-specific breast cancer families. *Nat Genet* 1996;13:120–2.
- Pinto M, Prise KM, Michael BD. Evidence for complexity at the nanometer scale of radiation-induced DNA DSBs as a determinant of rejoining kinetics. *Radiat Res.* 2005 Jul;164(1):73-85. PMID: 15966767
- Poplin E, Levy D, Berlin J, et al: Phase III trial of gemcitabine (30 min infusion) versus gemcitabine fixed-dose rate infusion (FDR) versus gemcitabine + oxaliplatin (GEMOX) in patients with advanced pancreatic cancer (E6201). *J Clin Oncol* 24: S18, 2006.
- Portugal J, Mansilla S, Bataller M. Mechanisms of drug-induced mitotic catastrophe in cancer cells. *Current Pharmaceutical Design* 2010;16:69–78.
- Potti A, Ganti AK, Tendulkar K, Chitajallu S, Sholes K, Koch M and Kargas S: HER-2/neu and CD117 (C-kit) overexpression in hepatocellular and pancreatic carcinoma. *Anticancer Res* 23: 2671-2674, 2003.
- Preston DL, Kusumi S, Tomonaga M, et al. Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950–1987. *Radiat Res.* 1994; 137(2 Suppl):S68–97.
- Pugliese R, Scandroglio I, Sansonna F, Maggioni D, Costanzi A, Citterio D, Ferrari GC, Di Lernia S, Magistro C. Laparoscopic pancreaticoduodenectomy: a retrospective review of 19 cases. *Surg Laparosc Endosc Percutan Tech.* 2008 Feb;18(1):13-8. PMID: 18287976
- Pui CH. Childhood leukemias. *N Engl J Med.* 1995;332: 1618–1630.
- Rai KR, Chiorazzi N. Determining the clinical course and outcome in chronic lymphocytic leukemia. *N Engl J Med.* 2003 May 1;348(18):1797-9. PMID: 12724486

- Rechsteiner M, Rogers SW PEST sequences and regulation by proteolysis *Trends Biochem Sci.* 1996 Jul;21(7):267-71. PMID: 8755249
- Redaelli A, Lee JM, Stephens JM, Pashos CL. Epidemiology and clinical burden of acute myeloid leukemia. *Expert Rev Anticancer Ther.* 2003;3:695–710.
- Reichard P. Ribonucleotide reductases: substrate specificity by allostery. *Biochem Biophys Res Commun.* 2010 May 21;396(1):19-23. Review.PMID: 20494104
- Reichard, P. Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.* 1988, 57, 349-374.
- Renaud S, De Lorgeril M. Wine, alcohol platelets, and the French paradox for coronary heart disease. *Lancet* 1992;339:1523–1526. [PubMed: 1351198]
- Robak T, Wierzbowska A . Current and emerging therapies for acute myeloid leukemia *Clin Ther.* 2009;31 Pt 2:2349-70. Review.PMID: 20110045
- Rosewicz S, Wiedenmann B. Pancreatic carcinoma. *Lancet*1997;15:786–92.
- Salucci M, Stivala LA, Maiani G, Bugianesi R, Vannini V. Flavonoids uptake and their effects on cell cycle of human colon adenocarcinoma cells (Caco2). *Br J Cancer* 2002;86:1645–1651. [PubMed: 12085217]
- Savitz DA, Andrews KW. Review of epidemiologic evidence on benzene and lymphatic and hematopoietic cancers. *Am J Ind Med.* 1997;31:287–295.
- Seymour JF, Huang P, Plunkett W, Gandhi V. Influence of fludarabine on pharmacokinetics and pharmacodynamics of cytarabine: implications for a continuous infusion schedule. *Clinical Cancer Research* 1996;2:653–8.
- Shao J, Zhou B, Chu B, Yen Y. Ribonucleotide reductase inhibitors and future drug design. *Curr Cancer Drug Targets.* 2006 Aug;6(5):409-31. Review.PMID: 16918309
- Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 1999 Jun 15;13(12):1501-12. Review. PMID: 10385618
- Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nature Reviews Cancer* 2003;3:155–68.
- Silverman DT, Dunn JA, Hoover RN et al. Cigarette smoking and pancreas cancer: a case-control study based on direct interviews. *J Natl Cancer Inst* 1994; 86(20): 1510–1516.
- Smith BD, Karp JE. Ribonucleotide reductase: an old target with new potential. *Leuk Res.* 2003 Dec;27(12):1075-6. PMID: 12921942
- Strasberg SM, Drebin JA, Soper NJ Evolution and current status of the Whipple procedure: an update for gastroenterologists *Gastroenterology.* 1997 Sep;113(3):983-94. PMID: 9287993

- Stubbe, J.; Nocera, D. G.; Yee, C. S.; Chang, M. C. Y. Radical initiation in the class I ribonucleotide reductase: long-range proton-coupled electron transfer? *Chem. Rev.* 2003, 103, 2167-2201.
- Sun J, Chu YF, Wu X, Liu RH. Antioxidant and antiproliferative activities of common fruits. *J Agric Food Chem* 2002;50:7449–7454. [PubMed: 12452674]
- Szekeres T, Fritzer-Szekeres M, Elford HL. The enzyme ribonucleotide reductase: target for antitumor and anti-HIV therapy. *Crit Rev Clin Lab Sci.* 1997 Dec;34(6):503-28. Review.PMID: 9439883.
- Szekeres T, Gharehbaghi K, Fritzer M, Woody M, Srivastava A, van't Riet B, Jayaram HN, Elford HL. Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase. *Cancer Chemother Pharmacol.* 1994;34(1):63-6.PMID: 8174204.
- Tanaka, H.; Arakawa, H.; Yamaguchi, T.; Shiraishi, K.; Fukuda, S.; Matsui, K.; Takei, Y.; Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* 2000, 404, 42-49.
- Taylor WR, Stark GR Regulation of the G2/M transition by p53. *Oncogene.* 2001 Apr 5;20(15):1803-15. Review.PMID: 11313928
- Thelander, M.; Graslund, A.; Thelander, L. Subunit M2 of mammalian ribonucleotide reductase. Characterization of a homogeneous protein isolated from M2-overproducing mouse cells. *J. Biol. Chem.* 1985, 260, 2737-2741.
- van den Heuvel S. Cell-cycle regulation. *WormBook.* 2005 Sep 21:1-16. Review.PMID: 18050422
- van der Donk, W. A.; Yu, G. X.; Perez, L.; Sanchez, R. J.; Stubbe, J.; Samano, V.; Robins, M. J. Detection of a new substrate-derived radical during inactivation of ribonucleotide reductase from *Escherichia coli* by gemcitabine 5'-diphosphate. *Biochemistry* 1998, 37, 6419-6426.
- Varmeh-Ziaie S, Manfredi JJ. The dual specificity phosphatase Cdc25B, but not the closely related Cdc25C, is capable of inhibiting cellular proliferation in a manner dependent upon its catalytic activity. *Journal of Biological Chemistry* 2007;282:24633–41.
- Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. *Cell Prolif.* 2003 Jun;36(3):165-75. Review.PMID: 12814432
- Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* 2003 Jun;36(3):131-49. Review.PMID: 12814430
- Wills PW, Hickey R, Malkas L. Ara-C differentially affects multiprotein forms of human cell DNA polymerase. *Cancer Chemotherapy and Pharmacology* 2000;46:193–203.

- Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. *J Agric Food Chem* 2003;51:609–614. [PubMed: 12537430]
- Xavier AC, Taub JW. Acute leukemia in children with Down syndrome. *Haematologica*. 2010 Jul;95(7):1043-5. Review. No abstract available. PMID: 20595099
- Yamaguchi, T.; Matsuda, K.; Sagiya, Y.; Iwadate, M.; Fujino, M.A.; Nakamura, Y.; Arakawa, H. p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. *Cancer Res*. 2001, 61, 8256-8262.
- Yamanaka Y, Friess H, Kobrin MS, Buchler M, Beger HG and Korc M: Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. *Anticancer Res* 13: 565-569, 1993.
- Yanamoto S, Iwamoto T, Kawasaki G, Yoshitomi I, Baba N, Mizuno A. Silencing of the p53R2 gene by RNA interference inhibits growth and enhances 5-fluorouracil sensitivity of oral cancer cells. *Cancer Letters* 2005;223: 67–76.
- Yao JC. Neuroendocrine tumors. Molecular targeted therapy for carcinoid and islet-cell carcinoma. *Best Pract Res Clin Endocrinol Metab*. 2007 Mar;21(1):163-72. Review. PMID: 17382271
- Yeo CJ, Cameron JL, Lillemoe KD, Sohn TA, Campbell KA, Hruban RH. Pancreaticoduodenectomy with or without distal gastrectomy and extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma, part 2: randomized controlled trial evaluating survival, morbidity and mortality. *Ann Surg* 236: 355-366, 2002
- Yeo CJ, Cameron JL. Pancreatic cancer. *Curr Probl Surg* 1999;36:59–152.
- Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. 2008 Jan;9(1):47-59. PMID: 18097445
- Zhang YW, Jones TL, Martin SE, Caplen NJ, Pommier Y. Implication of checkpoint kinase-dependent up-regulation of ribonucleotide reductase R2 in DNA damage response. *Journal of Biological Chemistry* 2009;284:18085–9
- Zhu KQ, Zhang SJ Involvement of ATM/ATR-p38 MAPK cascade in MNNG induced G1-S arrest. *World J Gastroenterol*. 2003 Sep;9(9):2073-7. PMID: 12970909

<http://www.cancer.gov>

<http://info.cancerresearchuk.org>

<http://www.atcc.org>

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## 10. Curriculum Vitae

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### PROFESSIONAL EXPERIENCE

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*Thesis: "Inhibition of ribonucleotide reductase by small molecules: A Key antitumor mechanism in human cancer cells"*
- 9/2006 – 01/2011** Research fellow at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna (Advisor: Prof. Dr. Thomas Szekeres)
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<b>2002 – 2003</b>	Studies of human medicine at the Medical University of Vienna
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## 11. Publications

1. Saiko P, **Graser G**, Giessrigl B, Lackner A, Grusch M, Krupitza G, Basu A, Sinha BN, Jayaprakash V, Jaeger W, Fritzer-Szekeres M, Szekeres T. A novel N-hydroxy-N'-aminoguanidine derivative inhibits ribonucleotide reductase activity: Effects in human HL-60 promyelocytic leukemia cells and synergism with arabinofuranosylcytosine (Ara-C). *Biochem Pharmacol.* 2011 Jan 1;81(1):50-9. Epub 2010 Sep 16. PMID: 20849828
2. Chetan B, Bunha M, Jagrat M, Sinha BN, Saiko P, **Graser G**, Szekeres T, Raman G, Rajendran P, Moorthy D, Basu A, Jayaprakash V. Design, synthesis and anticancer activity of piperazine hydroxamates and their histone deacetylase (HDAC) inhibitory activity. *Bioorg Med Chem Lett.* 2010 Jul 1;20(13):3906-10. PMID: 20605448
3. Saiko P, Ozsvár-Kozma M, **Graser G**, Lackner A, Grusch M, Madlener S, Krupitza G, Jaeger W, Hidvegi M, Agarwal RP, Fritzer-Szekeres M, Szekeres T. Avemar, a nontoxic fermented wheat germ extract, attenuates the growth of sensitive and 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells through induction of apoptosis. *Oncol Rep.* 2009; 21: 787-791. PMID: 19212640
4. Fritzer-Szekeres M, Savinc I, Horvath Z, Saiko P, Pemberger M, **Graser G**, Bernhaus A, Ozsvár-Kozma M, Grusch M, Jaeger W, Szekeres T. Biochemical effects of piceatannol in human HL-60 promyelocytic leukemia cells - synergism with Ara-C. *Int J Oncol.* 2008 Oct;33(4):887-92. PMID: 18813804.
5. Cappellacci L, Franchetti P, Vita P, Petrelli R, Lavecchia A, Jayaram HN, Saiko P, **Graser G**, Szekeres T, Grifantini M. Ribose-Modified Purine Nucleosides as Ribonucleotide Reductase Inhibitors. Synthesis, Antitumor Activity, and Molecular Modeling of N6-Substituted 3'-C-Methyladenosine Derivatives. *J Med Chem.* 2008 Jul 24;51(14):4260-9. Epub 2008 Jun 28. PMID: 18588281.
6. Saiko P, Ozsvár-Kozma M, Bernhaus A, Jaschke M, **Graser G**, Lackner A, Grusch M, Horvath Z, Krupitza G, Handler N, Erker T, Jaeger W, Fritzer-Szekeres M, Szekeres T. N-hydroxy-N'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxy-benzamidine, a novel resveratrol analog, inhibits ribonucleotide reductase in HL-60 human promyelocytic leukemia cells: Synergistic antitumor activity with arabinofuranosylcytosine. *Int J Oncol.* 2007 Nov;31(5):1261-6. PMID: 17912455