DIPLOMARBEIT

Comparison of the pro-apoptotic and anti-proliferative effects of two related CDK inhibitors

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Verfasserin / Verfasser:	Eva Walzi
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Betreuerin / Betreuer:	Univ.-Prof. Mag. Dr. Erwin Heberle-Bors

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Curriculum Vitae

EVA WALZI

Brunn 24 • 5201 Seekirchen • Austria • Telephone: (+43) 650 5547636 • ewalzi@gmx.at

Education:

Sept. 03 – present	University of Vienna: Study of 'Microbiology and Genetics') Diploma thesis: 'Comparison of the anti-proliferative and pro-apoptotic effects of two related cell cycle dependent kinase inhibitors'
Jan. 07 – June 07	Exchange to Lund, Sweden: Majors: Immunology and eukaryotic genetics
Sept. 01 – July 02	Evening school: For achievement of the Austrian 'Berufsreifeprüfung' (university entrance exam)
Sept. 00 – July 02	School for extended care and extended home care

Publications:

Wesierska-Gadek J., B. Hajek, Bettina Sarg, Stefani Wandl, **Eva Walzi**, Herbert Lindner 'Pleiotropic effects of selective CDK inhibitors on human normal and cancer cells';
Biochemical Pharmacology 76(2008) 1503-1524

'Outcome of treatment of human HeLa Cervical Cancer Cells with Roscovitine strongly depends on the dosage and cell cycle status prior to the treatment' by Jozefa Wesierska-Gadek, Andreea Borza, **Eva Walzi**, Vladimir Krystof, Margarita Maurer, Oxana Komina, and Stefanie Wandl
Journal of Cellular Biochemistry [in press]

Computer literacy:

Microsoft Office (MS Word, MS Exel, MS Power Point)
Corel Draw X3
Graph Pad Prism (for statistical analysis)

Abstract

Cell cycle progression is regulated by sequential and orchestrated activation of cyclin- dependent kinase (CDK)/cyclin complexes and additionally by cellular inhibitors of CDKs as well as by p53 protein, a product of a tumour suppressor gene. Deregulation of the cell cycle regulators as well as inactivation of intrinsic inhibitors of CDKs frequently occurring during malignant transformation constituted the rationale for development of pharmacological inhibitors of CDKs for therapy of cancers.

In the present thesis three structurally related tri-substituted purines, namely Olomoucine (OLO), Roscovitine (ROSC), and Olomoucine II (OLO II), were used for treatment of tumour cells. Whereas the action of the two first inhibitors of CDKs on different cancer types in experimental approaches and clinical trials was previously described, that of the latter, a very recently synthesised compound, is actually unknown. These tri-substituted purines, most ATP-related have been shown to be non-genotoxic and only slightly cytotoxic. These traits confer them an advantage over conventional cytostatic drugs and predestine them for therapeutic application. In the thesis the efficacy of the CDK inhibitors on two aggressive human cancer cell lines (HL-60 promyelocytic leukaemia cells and HeLa cervical carcinoma cells) and normal human fibroblasts (MRC-5 cells) was studied. Furthermore, to evaluate the role of the two in malignancy most frequently mutated genes, *TP53* and *RAS*, were tested in an appropriate experimental model. For this purpose cell clones overexpressing temperature-sensitive (ts) $p53^{135Val}$ mutant alone or in combination with mutated *c-Ha-Ras*, were used. The temperature sensitive $p53^{135Val}$ mutant offers the possibility to evaluate under the same genetic background whether the functional status of p53 (wild-type *versus* mutant) plays any role in the susceptibility of tumour cells to the anti-cancer therapy. Moreover, comparison of the outcomes of the treatment of immortalized cells (cells overexpressing $p53^{135Val}$) and transformed cells (cells overexpressing $p53^{135Val}$ + c-Ha Ras) allows to evaluate the role constitutive activation of pro-survival genes like *RAS* in the regulation of the sensitivity of cancer cells to growth-inhibitory and apoptosis-promoting drugs.

TP53 and *RAS*, the two genes most frequently mutated during malignant transformation, seem to determine the sensitivity of cancer cells to anti-cancer treatment and therefore, may be used as targets for therapeutic approaches.

TP53, encoding p53 tumour suppressor protein, called the 'guardian of the genome' (Lane, 1992) has numerous activities in cells. It is involved in the control of cell cycle, especially in

the regulation of checkpoints, DNA repair, senescence, and apoptosis thereby maintaining the genomic stability.

C-Ha-Ras, a small G-protein belonging to the *RAS* superfamily of genes, plays a key role in mitogens-dependent signalling. Mutations in *c-Ha-Ras* lead to its constitutive activation resulting in activation of downstream signalling cascades (e.g. MAP kinase pathway, PI3K pathway). The increased signal transduction provides an unregulated mechanism of proliferative stimulation and self-sufficiency in growth signals.

First, it was evidenced in my diploma thesis that OLO II, the newly developed CDK inhibitor had a strong anti-proliferative and pro-apoptotic potential that was comparable with or even exceeded that of ROSC. Secondly, it was shown that the effectivity of the used pharmacological CDK inhibitors on tested cancer cell lines markedly depended on the intrinsic status of p53 and c-Ha-Ras.

Unlike OLO, ROSC and OLO II have been shown to strongly inhibit proliferation of two investigated human cancer cell lines (HL-60 and HeLa cells) and of two immortalized rat cell clones (402/534, 602/534). In apoptosis prone human HL-60 promyelocytic leukaemia cells the *TP53* gene is disrupted. However, their checkpoint at the G_1/S border is intact. HeLa cervical cancer cells are HPV-18 positive and, as a consequence of the expression of the HPV-encoded oncoproteins E6 and E7, p53 protein as well as the G_1/S checkpoint are inactivated, respectively. The anti-proliferative effect was dosage- and time-dependent. Both inhibitors at lower dose generally arrest cell cycle progression and at higher concentration eliminate cells by apoptosis. Inhibition of cellular CDKs by ROSC or OLO II resulted in accumulation of the ratio of S- and/or G_2- phase cells in all tested cell lines implicating that the type of cell cycle arrest induced in asynchronously growing cells by CDK inhibitors does not depend on the functional status of the G_1/S checkpoint. Moreover, ROSC and OLO II induce the mitochondrial pathway of caspase-dependent apoptosis. Furthermore, the reduced susceptibility of transformed rat cells (189/111, 173/1022) to the CDK inhibitors indicates that overexpression of mutated c-Ha-Ras renders cancer cells insensitive or even resistant to the therapy. Finally, the status of p53 seems to be important for the outcome of the therapy by CDK inhibitors. ROSC is able to induce and even to reactivate p53 in HPV-positive cells. The up-regulated and activated p53 protein additionally promotes the growth-inhibitory and pro-apoptotic activities in treated cells.

Kurzzusammenfassung

Zellzyklusprogression wird durch die sequentielle und exakt koordinierte Aktivierung von Cyclin abhängigen Kinasen (CDKs), deren zugehörigen Cyclinen, von zellulären Inhibitoren der CDKs sowie vom Tumorsuppressorprotein p53 reguliert. Während der malignen Transformation kommt es zur Fehlregulierung von Zellzykluskomponenten und zur Inaktivierung von intrinsischen Inhibitoren von CDKs. Der oft beobachtete Verlust der zellulären CDK Inhibitoren in Krebszellen hat einige Forscher auf die Idee gebracht diese mit pharmakologischen Inhibitoren zu ersetzen. Dies führte zur Entwicklung von spezifischen CDK Inhibitoren, die in der Krebstherapie zunehmend eingesetzt werden. In meiner Diplomarbeit wurden drei miteinander verwandte dreifach substituierte Purine namens Olomoucine (OLO), Roscovitine (ROSC) und Olomoucine II (OLO II) für die Behandlung von Tumorzellen verwendet. Die Wirkungen der ersten beiden CDK Inhibitoren auf Krebszellen wurden bereits in mehreren experimentellen Modellen sowie auch in klinischen Studien gezeigt. Das Potential des erst kürzlich synthetisierten OLO II hingegen ist weitgehend unbekannt. Ein Vorteil von diesen drei dreifach substituierten Purine, der sie von konventionellen Cytostatikern unterscheidet, ist, dass sie nicht genotoxisch bzw.nur geringe cytotoxisch sind. Im Zuge dieser Diplomarbeit wurden die Effekte der oben angeführten CDK Inhibitoren auf zwei aggressive humane Krebszellen (HL-60 promyelozytische Leukemie und HeLa Gebärmutterhalskrebszellen) sowie auf die normalen humanen Fibroblasten (MRC-5 Zellen) evaluiert. Weiters verwendeten wir experimentelle Modelle, die es ermöglichten die Rolle der Gene *TP53* und *RAS*, welche häufig in bösartigen Tumoren verändert sind zu studieren. Dafür wurden Zellklone, die temperatur sensitives (ts) $p53^{135Val}$ allein, oder in Kombination mit mutiertem *c-Ha-Ras* expremieren, verwendet. Diese Zellklone mit temperatur-sensitivem p53 ermöglichten es uns die Auswirkungen vom p53 Funktionsstatus (wild typ *versus* mutant) auf die Effizient von Anti-Krebstherapien in Zellen von gleichem genetischen Hintergrund zu studieren. Weiters erlaubte der Vergleich von Behandlungsergebnissen von immortalisierten Zellen (Überexpression von $p53^{135Val}$) mit denen von transformierten Zellen (Überexpression von $p53^{135Val}$ + c-Ha-Ras) die Ermittlung des Einflusses von konstant expremierten pro-survival Genen wie z.B. *RAS* auf die Effizient von Krebstherapien. *TP53* und *RAS,* zwei Gene die häufig während der malignen Transformation mutiert werden scheinen ausschlaggebend für die Sensitivität von Krebszellen für Therapie zu sein und stellen daher wichtige Ziele in der modernen Krebsforschung dar. *TP53,* welches für das Tumorsupressorprotein p53 kodiert, wird aufgrund der vielfältigen

Aufgaben des Proteins in der Zelle auch “Wächter des Genoms” (Lane, 1992) genannt. Das p53 Protein ist in der Zellzyklusregulation - speziell bei der Regulation von Checkpoints - involviert und bekleidet weiters Funktionen in DNA Reperatur, Senescence und Apoptose. Auf diese Weise, erhält das Protein die genomische Stabilität. *C-Ha-Ras*, ein kleines G-Protein welches zur Familie der *RAS* superfamilie gehört spielt eine tragende Rolle in nährstoffabhängigen Signalwegen. Mutationen, die zur konstitutiven Aktivierung von *c-Ha-Ras* und somit seiner downstream Signalwege (z.B. MAP Kinase Signallweg, PI3K Signalweg) führen, ermöglichen unkontrollierte Proliferation und von wachstumsfaktoren-unabhängiges Wachstum. Während meiner Diplomarbeit konnten wir feststellen, dass OLO II, der kürzlich entwickelte CDK Inhibitor, genauso starke oder vielleicht sogar stärkere anti-proliverative und pro-apoptotische Wirkungen zeigt wie ROSC. Weiters konnten wir nachweisen, dass die Effektivität der getesteten pharmkologischen CDK Inhibitoren tatsächlich stark vom intrisischen p53 und *c-Ha-Ras* Status abhängt. Im Gegensatz zu OLO können OLO II und ROSC die Proliferation der humanen Krebszelllinien HL-60 und HeLa sowie die der beiden immortalizierten Rattenzelllinien (402/534, 602/534) hemmen. In den zur Apoptose neigenden humanen Leukämiezellen HL-60 ist *TP53* inaktiviert. Trotzdem ist der G_1/S checkpoint in dieser Zelllinie intakt. Im Gegensatz dazu, ist p53 und der G_1/S Checkpoint in den HPV-18 positiven HeLa Gebärmutterhalskrebszellen aufgrund von den HPV-18 kodierten Oncoproteinen E6 und E7 inaktiv. In beiden Zelllinien konnten wir nachweisen, dass die anti-proliferative Wirkung zeit- und konzentrationsabhängig ist. In Folge von niedrigen OLO II oder ROSC Konzentrationen wurde Zellzyklusarrest induziert, höheren Konzentrationen hingegen führten zur Elimination der Zellen mittels Apoptose. Die Hemmung der zellulären CDKs durch ROSC oder OLO II führte zur Vermehrung der Zellpopulationen in der S- oder G_2- Zellzyklusphase in allen verwendeten Zelllinien. Dies deutet darauf hin, dass die Art des Zellzyklusarrests - welcher in asynchron wachsenden Zellen durch CDK Inhibitoren induziert wird - nicht vom Funktionsstatus des G_1/S checkpoint abhängig ist. Weiters induzieren ROSC und OLO II den mitochondrialen Signalweg der Apoptose. Das verminderte Therapieansprechen von den transformierten Ratenzellen (189/111, 173/1022) lässt vermuten, dass die Überexpression von mutiertem c-Ha-Ras dazu in der Lage ist, Krebszellen insensitiv oder sogar resistent gegenüber CDK Inhibitoren zu machen. Der p53 Status scheint wichtig für das Therapieergebnis mit CDK Inhibitoren zu sein. ROSC ist dazu in der Lage p53 in HPV-positiven Zellen zu reaktivieren. Dieses Protein führt in Folge zu Wachstumshemmung und pro-apoptotischen Effekten in behandelten Zellen.

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1. INTRODUCTION

1.1 Cancer

On December 23, 1971, Nixon – president of the United States at this time - signed a National Cancer Law to make the ‘conquest of cancer’ a national crusade, hence declaring war on cancer. Decades later cancer is still one of the worlds widest distributed malignancies.

Under normal circumstances all cells have an ending lifespan. At the end of this lifespan cell death occurs either via necrosis (cell death after cell damage by external force) or by apoptosis (a regulated process of ‘cell suicide’). [1] By the means of apoptosis the body is able to eliminate damaged or unnecessary cells hence preventing uncontrolled cell growth without local inflammation. This control of cells growth is a fundamental process involved both in human embryonic development and adult tissue homeostasis. [2]
But if proper cell growth control – among other parameters - is no longer maintained this can lead to transformation of normal human cells into malignant cancer cells. [3] Alterations in the genome of a cell ranging from subtle sequence changes (e.g. base substitution or insertion of a few nucleotides) alteration in chromosome number (e.g. losses or gains of whole chromosomes) to chromosome translocations (e.g. fusions of different chromosomes) are what promote this uncontrolled growth. [4] Obviously a single mutation is not sufficient to induce cancer development because otherwise every human being would suffer from several tumours. For tumour formation several genomic alterations (=hits) have to take place. [5] Even in that case cancer development is not necessarily the consequence. Only a certain set of mutations – mainly in regulatory genes - does indeed promote cancer.

1.1.1 Hallmarks of cancer

Douglas Hanahan and Robert A. Weinberg proposed six changes in cell physiology that together dictate malignant growth (**Fig.1**). [3]

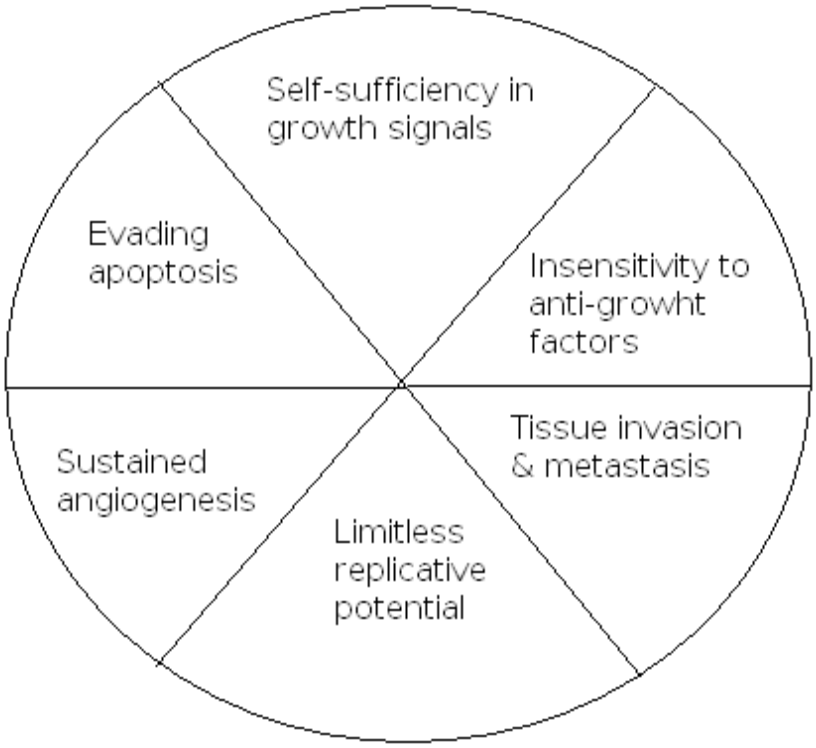

Figure 1. Hallmarks of cancer

Self-sufficiency in growth signals

Normal human cells require growth signals conferred via complex signalling pathways for growth. If no growth factors are present normal cells are therefore incapable to proliferate. In malignant cells on the other hand the pathways that contribute to normal and physiologic actions of growth factors are often altered [6] ultimately leading to growth factor independent cell growth. Hence one characteristic of cancers is loss of cellular growth control. [7]

Insensitivity to growth-inhibitory signals

Growth inhibitors e.g. TGF β1 in epithelial cells ensure that tissue homeostasis is maintained. A lot of growth inhibitors - among them TGF β1 - inhibit cell cycle progression of some cells by preventing hyperphosphorylation of Retinoblastom etc. while they allows the cell cycle progression of other cells (mechanism unknown). This balance maintains tissue homeostasis.
In carcinogenic cells this growth inhibition is often lost due to loss of signalling components or because of mutations of downstream components (e.g. Ras activations in the case of TGF β1). [8]

Evasion of programmed cell death (apoptosis)

Altered expression or mutations of genes encoding for crucial apoptotic proteins help malignant cells to evade apoptosis. Examples would be the over expression of anti-apoptotic proteins such as BCL-2 or the down-regulation of the death receptor Fas. [9]

Limitless replicative potential

Nontransformed cultured cells have a limited replicative capacity. Hence cells enter senescence after a defined number of generations (Hayflick limit). [10]

The main reason why cells can't replicate forever is that their telomeres are shortening with every replicative event (the so called telomere end-replication problem). [11] The enzyme telomerase – a reverse trancriptase that is able to extend telomeres - is normally only active in germ line cells but not in somatic cells. By reactivation of telomerase chancerogenic cells can circumvent the telomere end-replication problem and keep replicating. Telomerase is elevated in a vast majority of tumours. [12]

Sustained angiogenesis

Angiogenesis (the formation of new blood vessels) is a normal process in growth and development and is necessary to supply tissues with nutrient and oxygen and to remove waste products. Angiogenesis is also required for tumour growth and spreading.

The development and maturation of blood vessels is regulated by a complex network of pro- and antiangiogenic factors. If the balance of this network is deregulated tumourogenic blood vessels can form. [13]

Tissue invasion and metastasis

Tumour cells can spread into surrounding tissues, invade blood vessels and leave the blood stream at different sites by interaction with extracellular matrix components and epithel-mesenchymal transition. Angiogenesis is a prerequisite to enable tumour cells to do so. [14]

1.1.2 Molecular abnormalities leading to cancer

Genetic mutations (inversions, substitutions, insertions, deletions, translocations etc.) are largely responsible for the generation of malignant cells. Mutations promoting tumour growth are often mutations taking place in genes for proteins that regulate cell growth, division and DNA repair. Oncogenes and tumour suppressor genes are a subset of genes that are frequently mutated in cancer.

1.1.2.1 Oncogenes

Oncogenes are sequences of DNA that have been altered (e.g. overexpression) or mutated from their original form the proto-oncogene. [15] Genes that turn into oncogenes that promote transormation of a normal cell into a cancer cell are mainly regulatory genes (e.g. transcription factors) or members of important signal transduction pathway. Their functions in a non mutated/non-overexpressed state range from functions in cell divisions to functions in regulation of apoptosis hence the effects of mutations in those genes are very wide spread. The consequences of mutations are often unregulated cell division or/and growth in the absence of growth signals both characteristics of a tumour cell.

1.1.2.1.1 Ras (for rat sarcoma virus)

Small GTPases are proteins of 20-29 kDa that are characterised by their ability to bind and hydrolyse GTP (see bottom). All of them share common features like sequence and biochemical similarities [16] but have different functions in a cell. Harvey Ras (Ha-Ras), Kirsten Ras (K-Ras) and N-Ras are prominent and probably the best studied members of the at least 35 closely related human Ras proteins (**Fig. 2**).

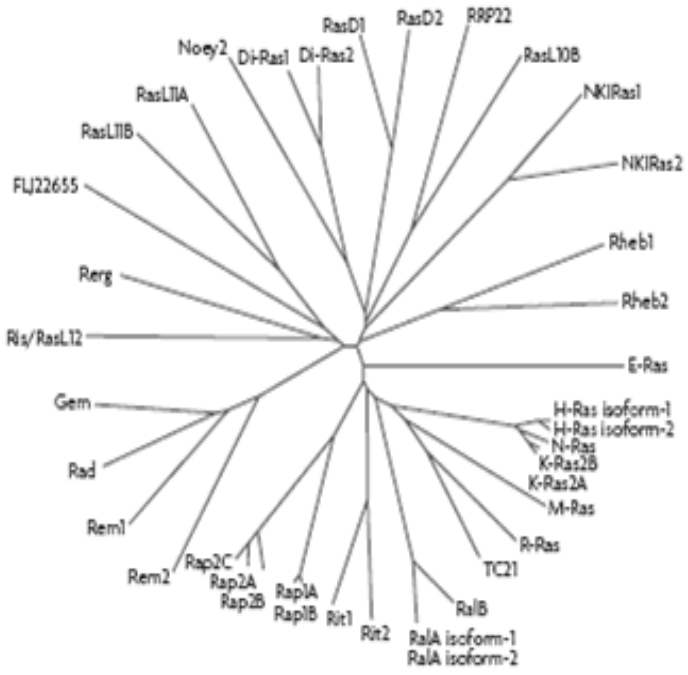

Figure 2. The Ras family

(Graph by: Antoine E. Karnoub and Robert A. Weinberg see reference 18)

If a broader definition of sequence similarity is used the Ras family counts 170 members (subclassified into Ras, Rho, Rag, Arf and Ran families). [17] H-Ras (Harvey sarcoma virus-associated oncogene) and K-Ras (Kirsten sarcoma virus) came to their names because of their viral homologues in rat sarcomas and are of great scientific interest because of their frequent mutations in human cancer cell lines. [18] The third member of the Ras-related genes N-Ras is also found to be mutated in human tumours e.g. melanomas. [19] These three Ras proteins have overlapping but still different functions in mammalian cells. [20]

H-, K-, and N-Ras will henceforth be collectively referred as Ras.

1.1.2.1.1.1 Ras signal transduction

Ras proteins are membrane anchored components (see posttranslational modifications) of signalling cascades ultimately leading to activation of various downstream targets (activation of those results in cellular proliferation or differentiation, depending on the cellular background). Activation of Ras proteins can happen by different extracellular stimuli e.g. growth factors or cytokines via signals from cell-surface receptors. For many growth factors or cytokines to induce nonproliferative cells to enter the G_1 phase of cell cycle the activation of Ras by binding of GTP and hydrolysis of the same is essential. [21]

Activation of Ras is the result of the stimulated exchange of GDP for GTP catalysed by a guanine nucleotide-exchange factors (GEFs), such as SOS (son of sevenless) in the case of Ras. GEFs lead to the release of GDP from the GTPase through interaction with the switch regions (I and II) and the phosphate-binding loop of the GTPase. [22] The GEFs responsible for GDP release used in the distinct Ras subfamilies are structurally distinct but share common mechanistic features. [20] In the case of growth factor dependent Ras activation the so called GRB2 protein (growth factor receptor-bound protein-2) is additionally required as an adaptor that associates with the EGF (epidermal growth factor) receptor that is a tyrosine kinase. GRB2 concomitantly attaches to mammalian SOS. The GRB2-SOS complex can attach to the receptor tyrosine kinase directly or via an adaptor protein such as SHC. [23] Inactivation of Ras involves GAPs (GTPase activating proteins). Ras and other small GTPases are very weak GTPases and therefore require the 'help' of GAPs to catalyse GTP hydrolysis. GAPs greatly speed up the hydrolysis of GTP by binding to Ras via their C-terminus. [24] Both GAP and GEFs were found to contain so called Src-homology-2 or 3 (SH2/SH3) motifs for interaction with other regulatory partners.

The exact mechanism that leads to Ras activation and inactivation is still under debate but involves conformational changes mainly in switch I and II that are required for interaction of Ras with its up- and downstream partners. [18]

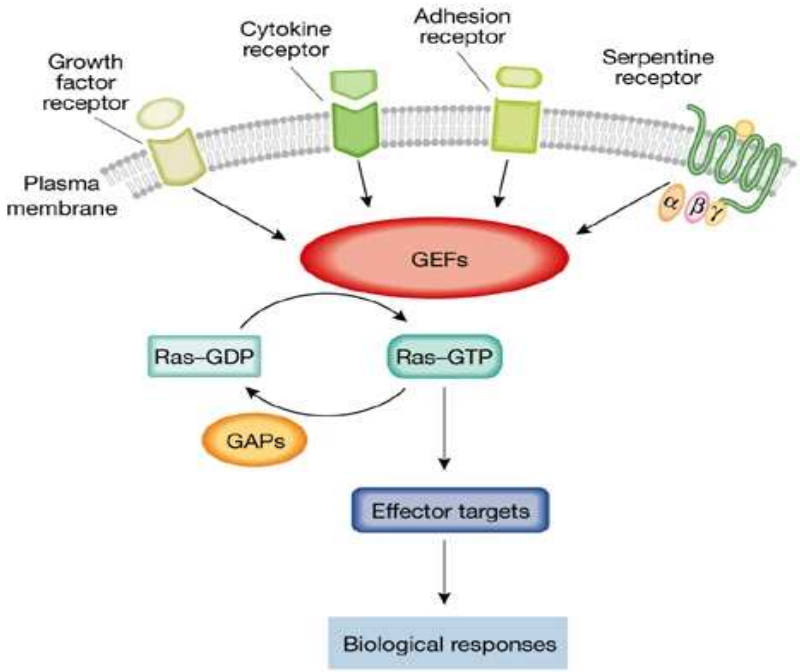

Figure 3. Ras signalling cascade

(Graph by Yi Zheng and Lawrence A. Quilliam see reference 20)

1.1.2.1.1.2 Downstream targets of Ras

Once activated Ras is able to regulate a number of proteins involved in distinct signalling pathways. Among them members of the Raf family (Raf1, B-raf and A-raf), phosphatidylinositol 3 kinase (PI3K) and members of the RalGEF family (RalGDS, Rlf and Rgl).

The serine-threonine kinase pathway Raf-MEK-ERK is probably the most studied downstream pathway of Ras. [25]

Raf-MEK-ERK pathway

Mitogen-activated protein (MAP) kinases ERK1 (extracellular signal-regulated kinase 1) and ERK2 are both activated by Ras in response to mitogen stimulation. To do so Ras directly binds to Raf which will recruit the MAP kinases and their subordinates. [26]

Activated MAP kinase in turn phosphorylates transcription factors such as c-Fos that are required for progression through early-G1 phase of the cell cycle. [21]

p38 and JNK (c-JUN NH_2-terminal protein kinase) pathway

p38 and JNK are MAP kinases just like ERK1/2 but are induced by cellular stress instead of growth factors and do not need Raf for their signal transduction cascade. The dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways is maybe important in determining whether a cell survives or undergoes apoptosis. [27]

Phosphatidylinositol 3 kinase signal transduction

The extracellular stimuli that are required for Ras activation prior to PI3K activation can be of various sources e.g. IL-3, PDGF. [25] Once activate PI3K phosphorylates inositol lipds which are second messengers for – amongst others – the Akt/PKB kinase. [28] After complete activation of Akt/PKB by phosphorylation it phosphorylates BAD a pro-apoptotic protein of the Bcl-2 family amongst other targets. Phosporylation of BAD leads to its inactivation hence inhibition of apoptosis. [29]

1.1.2.1.1.3 Post-translational modifications of Ras

Ras undergoes a series of post-translational modifications including farnesylation, methylation and palmitoylation at the C-terminal CAAX motif (C = cysteine; A = aliphatic; X = any amino acid).

The first modification to take place is farnesylation by the enzyme farnesyltransferase (FTase) followed by proteolytic cleavage of the AAX sequence by RCE1 (Ras-converting enzyme-1). The remaining cystein is then methylated by the isoprenylcysteine carboxymethyltransferase-1 (ICMT1). [18] For membrane localisation another modification namely a palmitoylation is required. Nonpalmitoylated but farnesylated and methylated mutants mislocalizes to the cytosol and fails to promote maturation. [30]

All of the above mentioned posttranslational modifications are important not only for Ras localization in the membrane but also for its interaction with its effector proteins. [31]

1.1.2.1.1.4 Ras in cancer

Constitutively activating Ras mutations, mutations in its downstream targets or in cell surface receptors involved in Ras signalling have been identified in many human tumours. [32]

1.1.2.2 Tumour suppressors

Tumour suppressors are protective genes that normally limit the growth of tumours by various means e.g. induction of apoptosis in DNA damaged cells hence they prevent the action of oncoproteins. If a tumour suppressor gene is mutated (altered), it may fail to keep a cancer from growing.

1.1.2.2.1 p53

p53 is a transcription factors involved in cell cycle regulation, apoptosis, DNA repair, senescence, and angiogenesis. [33] Because of its wide area of function it was termed the 'Guardian of the Genome' [34] and even voted 'Molecule of the Year' in 1993.
p53 consists of a N-terminal acidic transactivation domain (TAD), a proline rich region, a DNA binding region and a tetramerization domain. [35] TAD is a binding site for a variety of interaction partners of p53 such as the transcription machinery [36], transcriptional co-activators such as p300/CBP (CREB-binding protein) [37] and MDM2 a negative regulator of p53 transcription activity.
The DNA binding region is required for binding of p53 target DNA sequences. It is subdivided into two structural motifs that bind the minor/major groove of target DNA sequences. [35] The C-terminal tetramerization domain of p53 is required for oligomerization of the protein as p53 can only interact with DNA as a tetrameric structure.

1.1.2.2.1.1 p53-MDM2-ARF interaction

In resting cells p53 is expressed at a very low levels and its half-life does usually not exceed 30 minutes. Only after exposure to DNA damaging agents or other stress stimuli (e.g. aberrant growth signals or chemotherapeutic drugs) [38] p53 is stabilized and activated by posttranslational modifications. [39]
The reason why p53 is only present at low levels in unstressed cells is targeted degradation of p53 protein mediated by MDM2 a negative regulator of p53. [40]

MDM2 regulates p53 in three different ways: [41]

- inhibition of its transcriptional activity by binding to the TAD
- export of p53 out of the nucleus hence separating it from its target genes
- promoting proteasome-mediated degradation by functioning as an E3 ubiquitin ligase (**Fig. 4**)

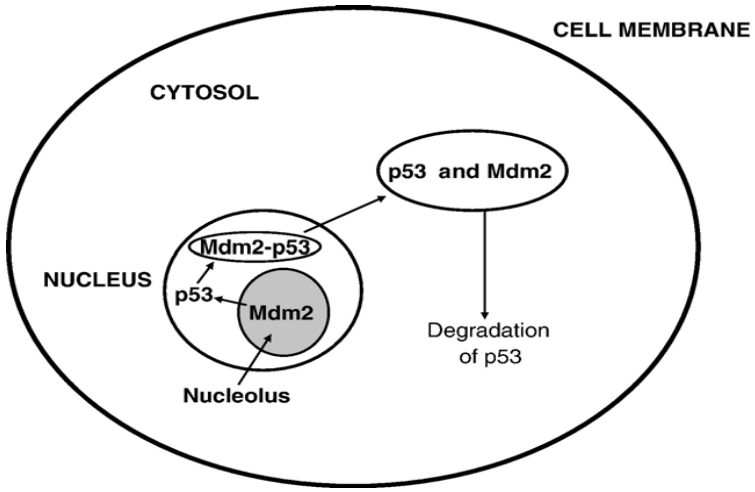

Figure 4. p53 regulation by MDM2

(Graph by: S. Shangary, S. Wang see reference 41)

MDM2 itself is regulated by p53-MDM2 and p14 ^{ARF}-MDM2 feedback loops. [38] p53 regulates MDM2 expression by binding to a p53 binding site in the first intron of MDM2 hence p53 leads to the transcription of its own negative regulator (auto-regulatory feedback loop). [42] By posttranslational modifications of p53 and Mdm2 (see bottom) and binding of the protein p14 ^{ARF} to MDM2 the interaction of p53 and MDM2 is altered in response to DNA-damage allowing p53 to induce its target genes. [43] p14 ^{ARF} regulates Mdm2 by preventing the transportof p53 form the nucleolus to the cytoplasm thereby preventing Mdm2 mediated degradation of p53. [44]

1.1.2.2.1.2 Posttranslational modifications of p53

In response to stress signals p53 is target by various posttranslational modifications ranging from phosphorylation at various serine and/or threonine (e.g. Ser20, Thr81) residues to acetylation, sumoylation, methylation and polyadenylation. [45] The consequence of these

modifications is often the abrogation of the p53-Mdm2 interaction and/or the stabilization of p53. [39]

Phosporylation is mediated by various kinases probably depending on the kind of cellular stress. [46] Examples for those kinases are ATM, ATR, Chk1, Chk2, JNK and p38. The most frequently occurring phosporylation is phosphorylation of Ser 15 in response to different stresses e.g. DSB. [47]

Acetlyation on p53 occurs at 5 C-terminal lysine residues of p53 in response to cellular stress. It often enhances the DNA-binding activity of p53 in vitro, and is mentioned in context of p53 stabilization. [48] One protein that is known to acetylate p53 is p300 which is a transcriptional coactivator of p53. [49]

The effects and whereabouts of methylations and sumoylations of p53 are less well studied.

One more way to activate p53 is the addition of poly (ADP-ribose) moieties (polyadenylation) by PARP-1. PARP-1 is activated in response to single or doublstrand breaks and leads to rapid stabilization of p 53 [50]

1.1.2.2.1.3 Functions of p53

As already mentioned p53 is a transcription factors involved in various processes among them cell cycle regulation, apoptosis, DNA repair, senescence, and angiogenesis.

p53 in cell cycle arrest

Cell division is among the tightest regulated processes in a cells. [51] To ensure that the complete genome is faithfully transmitted from parent to daughter several checkpoints – G1/S, intra-S, G2/M, spindle checkpoint - are maintained. p53 is directly involved in several checkpoints especially in the G1/S checkpoint [52] (see bottom).

Induction of apoptosis by p53

In order to ensure that the apoptotic program is well-coordinated once the process is initiated p53 controls several component of the apoptotic cascade. It is involved in the intrinsic and the extrinsic pathway of apoptosis (see bottom) and can induce apoptosis in transcription-dependent and –independent fashion. [53]

Apoptosis vs. cell cycle arrest

How p53 decides to induce cell cycle arrest in one case and apoptosis in another case is subject of investigation and has not yet been resolved. It is indicated though that this decision

could be p53 level dependent. High levels of p53 leading to apoptosis and low levels to cell cycle arrest respectively. [54] Another possibility would be that the affinity of p53 binding sites within target gene promoters (high or low affinity binding sites) determines whether cell cycle arrest or apoptosis takes place. [55]
One protein apparently involved in this decision is Hzf (hematopoietic zinc finger), a protein that binds to the DNA binding domain of p53 after mild damage preferential leading to p53 mediated expression of genes involved in cell cycle arrest e.g. p21, 14-3-3 whereas pro-apoptotic genes e.g. Puma or Bax are diminished. If the damage extends Hzf is proteolytic degraded freeing p53 so that it can activate pro-apoptotic genes. [56] It is as yet unclear how Hzf directs p53 target gene selection or if it actively or passively represses pro-apoptotic gene expression. [55]

p53 in the regulation of angiogenesis
One of the newly discovered functions of p53 is limitation of angiogenesis by at least three mechanisms: [57]

1. interfering with central regulators of hypoxia that mediate angiogenesis
2. inhibiting production of proangiogenic factors
3. directly increasing the production of endogenous angiogenesis inhibitors

Angiogenesis is the normal process of new blood vessel formation which constantly occurs during tissue development but does not occur frequently in adulthood (only during wound healing or placenta formation). [57] As angiogenesis is critical for tumour formation (supplement of oxygen and nutrients) limitation of angiogenesis provides one more important way of p53 tumour control. [58] As a consequence tumours with p53 mutations/inactivation turn out to be more vascularised and often more aggressive. [57]

1.1.2.2.1.3 p53 in cancer

Functional impairment of p53 occurs in nearly 50% of human cancers. [59]
As p53 displays various functions in a cell, disruption of p53 function promotes a multitude of effects ranging from checkpoint defects, cellular immortalization, genomic instability and inappropriate survival that allows continued proliferation and evolution of damaged cells. [53]

1.1.2.3 Ras and p53

Next to their functions in the cell cycle p53 (see cell cycle arrest vs. apoptosis etc.) and Ras (growth factor dependent signalling) are involved in many other cellular pathways that will not be mentioned here due to space limitations. For this reason it appears only logic that those molecules are connected in one way or another. One example for their interaction concerning the cell cycle is mediated by the protein Survivin (see bottom) a protein that is p53 and/or Rb dependent but can also be influenced by Ras and that is upregulated in a vast number of tumours.

1.2 Cell cycle

The cell cycle is a fundamental process taking place in a cell to ensure the fidelity of the transmission of genetic information, correct DNA replication and equal segregation of genetic material during mitosis. In short, it maintains the stability of the genomic DNA over generations. [46] It is subdivided into four cell cycle phases of different function (**Fig. 5**):

1. G1 (gap phase 1)
2. S (DNA synthesis)
3. G2 (gap phase 2)
4. M (mitosis)

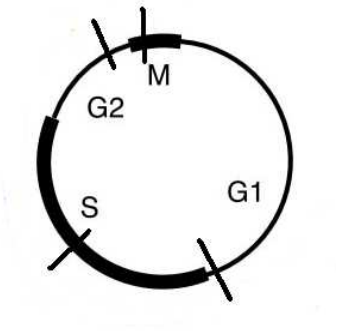

Figure 5. Cell cycle and checkpoints

Cell cycle progression is maintained by transient activation or inactivation of cell cycle dependent kinases (CDKs) by their association with different cylins and/or cyclin dependent kinase inhibitors (see bottom) and a few modifications. In active state CDK-cyclin complexes phosphorylate a series of substrate proteins promoting the events of the cell cycle. Three basic mechanisms are involved in this regulation: [60]

1. CDKs alone are catalytically inactive
2. the binding of different cyclins, which are required to activate CDKs determine the set of substrate proteins phosphorylated
3. the activity of cyclin-CDK complexes can be modulated by post synthetic modifications of the CDKs, or by binding of specific inhibitors

1.2.1 Main players in the cell cycle: CDKs and Cyclins

Two main components of the cell cycle that are involved in every step of it are cell cycle dependent kinases (CDKs) and cyclins which form heterodimeric complexes constisting of a catalytic kinase subunit and a regulatory cyline subunit. [61]

Cyclins

As their name already suggests cylins are expressed in a periodical, cell cycle phase dependent fashion (**Fig.6**). [50] This is necessary because the concentration of the catalytically inactive cyclin-dependent kinases is fairly constant during the cell cycle and their substrate specificity is hence determined by the exchange of the regulatory cyclin subunits. Binding of a CDK to a specific cyclin is determined by cyclin availability which is the result of equilibrium between the rates of their synthesis and degradation. [60]

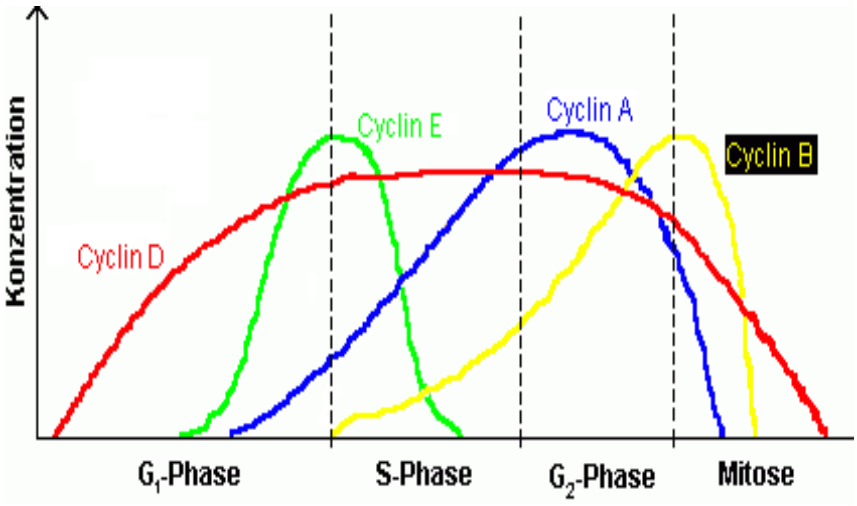

Figure 6. Periodicity of cyclin expression (graph by wikimedia)

Cell cycle dependent kinases

In contrast to cyclins CDKs are expressed in a fairly constant fashion throughout the cell cycle hence require regulation by other means than controlled synthesis and degradation. [62] As expected the activities of these proteins are regulated by a multitude of mechanisms including phosporylation of the CDKs by CDK activating kinase in complex with cyclin H (CAK) (see bottom), binding to the proper/available cyclin, removal of inhibitory phosphates and by cell cycle inhibitors (CDKIs) such as p21 or p27. [50]

CDKs are devided into two subgroups. Those involved in cell cycle progression (e.g. CDK 1, 2, 4) and those involved in transcriptional regulation (e.g. CDK 7, 8, 9). [63]

The transcriptional active CDKs like CDK7 in complex with cyclin H, CDK8 in complex with cyclin C and CDK9-cyclin T promote initiation and elongation of nascent RNA transcripts by phosphorylating the carbox-terminal domain of RNA polimerase II. [64]

The CDK7-cyclin H complex is further reported to be active in cell cycle progression and basal transcription hence providing a link between transcription and cell cycle regulation. [46]

1.2.2 Cell cycle progression

Initiation of the cell cycle progression is critically dependent on the accessibility of growth factors like the D type cyclins (D1, 2, 3) that are transcribed via a Ras etc. mediated pathway in response to growth factors stimulation. Subsequently cyclin D and CDK4 or 6 are able to bind each other promoting CDK4/6 activity (phosphorylation by CAK is further required for full activity). [65] Cylin D is a very short lived protein (~39 min) hence if growth factors are no longer available cyclin D will cease to be expressed and activation of CDK4/6 will stop. This provides one way to ensure that the cell cycle does not start without the proper supplies. [63] The third G_1 CDK –that is activated later than CDK4/6 - is CDK2 that needs to form a complex with cylin E in order to become partially activated. Phosporylation of threonin 160 of CDK2 by CAK. [66] and removal of inhibitory phosphates by Cdc25 phosphates are further required for full activation. Upon activation all three CDK-cyclin complexes start to phosphorylate the tumour suppressor protein Rb (Retinoblastoma protein).

At the beginning of G_1 Rb exists in a hypophosporylated state that allows it to bind members of the E2F transcription factor family [46] which are thereby rendered inactive hence unable to activate their target gene that would promote cell cycle progression.

If hyperphosporylated by CDK4/6-cyclin D and subsequently CDK2-cyclin E, Rb is no longer able to bind E2F thereby freeing the transcription factor. As E2F transcription factors regulate the expression of a number of genes important in cell proliferation, particularly those involved in progression through G_1-and into the S-phase of the cell cycle, cell cycle progression will be allowed [67] if no stress stimuli were detected.

One E2F target is cyclin A that is required and rate limiting for entry into S phase in mammalian fibroblasts. [68] Expression of the cyclin A gene in nontransformed cells is characterized by repression of its promoter during early G_1 phase of the cell cycle and its induction at late G_1 phase. [69]

Cylin A in complex with CDK2 is required for G_1/S transition, DNA replication and G_2 progression. [70] Cylin B the cyclin that is required in G_2 and M phase of the cell cycle is transcribed at a basal level throughout the cell cycle but its mRNA level increases strongly during G_2 phase to four times the amount present in G_1 [71] which allows the accumulation of cyclin B-CDK1 complexes. [72] To avoid improper M phase entry the cyclinB-CDK1 complex is kept in an inactive state by inhibitory phosphates at T14 and Y15 by Wee and Myt kinases [73] until dephosphorylation of the complex by phosphatases of the Cdc25 family takes place. [74] Dephosphorylation is induced by spindle checkpoint mediated signals (see bottom).

Ultimately the destruction of cyclin B via the polyubiquitination pathway [75] is required for the inactivation of the complex and exit from mitosis. [76] This destruction is initiated by the anaphase-promoting complex which ubiquitinates cyclin B at the metaphase–anaphase transition. [77]

1.2.3 Checkpoints

As already mentioned the activities of CDKs are controlled by a variety of mechanisms and a group of molecules that inhibit CDK activity the so called CDK inhibitors (CDKIs). [78] Those CDKIs are involved in cell cycle checkpoints ensuring that events such as DNA replication and chromosome segregation are completed with high fidelity and that the timing of cell cycle transition is correct.

G_1/S: The G_1/S checkpoint is required to ensure that cell cycle progression is inhibited if the cell is not provided with a sufficient amount of nucleotides, the polymerases is inhibited or DNA damage occurred. [79] The kinases ATM/ATR, CHK1/CHK2 and protein p53 play major roles in this checkpoint. In response to DNA damage by ionizing radiation ATM/ATR are activated and in turn activate CHK1/CHK2 by phosphorylation. Upon activation CHK1/CHK2 inactivate and destabilize Cdc25 phosphates and thereby inhibit CDK2 due to inhibitory phosphates at Tyr15. Furthermore CHK1 activates p53 [80] via phosporylation in its tranactivation domain (mainly on S15) [81] which in turn leads to transcription of the CDK complex inhibitor p21. Moreover p21 can associate with the proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA polymerases and resulting in both G1 and G2 arrest. [82]

Further p53 can inhibit the translation of CDK4 mRNA hence decreases the protein level of CDK4 which is required for G_1/S progression (see bottom). [83]

intra-S: The intra-S phase checkpoint is still poorly understood and under current investigation. So far it has been established that the p53-p21 pathway is likely activated in the intra-S checkpoint but that this pathway is not equally effective in response to different kinds of DNA damage [84, 85]

G_2/M: The G_2/M checkpoint ensures that mitosis is complete, growth and protein synthesis are efficient and no DNA damage occurred. Several p53 transcriptional targets are involved in the G_2/M checkpoint. p21 inhibits CDK1 directly, 14-3-3σ anchors CDK1 to the cytoplasm

where it cannot induce mitosis and Gadd45 that dissociates CDK1 from cyclin B1. The repression of either cyclin B1 or CDK1 enforces cell cycle arrest. Additionally p53 has other targets that induce G_2/M arrest independent of CDK1 [52]

spindle checkpoint: The spindle checkpoint is necessary to monitor correct attachment of kinetochores to microtubules so that sister chormatids are separated correctly and only if no defect was detected. The spindle checkpoint is mainly promoted by APC (anaphase – promotion – complex) which is activated by Cdc20 only if no damage was detected. Defects or presence of unattached kinetochores will result in the inactivation of APC via proteins such as Mad1/2, Bub1/2. [79] If no defects were detected Cdc20-APC phosporylate cyclin B and lead to its ubiquitination. Further APC- Cdc20 cleaves the inhibitory chaperonin securing which has kept the phospatase separase in an inactive state. [86] In an active form separase will cleave of the cohesion rings that maintain sister chormatid cohesion. [87] Sister chormatid separation hence progression in mitosis will be possible after cohesion cleavage. Failures of this checkpoint can result in cells exiting mitosis and entering the next S phase with a 4N DNA content in endoreplication. [79] If cells happen to evade the spindle checkpoint by this so termed 'mitotic slippage' p53 is activated and leads to a G_1-like growth arrest. Due to this cells that have an intact nucleus but contain 4N DNA will be prohibited to devide. Cells lacking p53 will still transiently arrest in mitosis and fail to divide but are not prevented from re-entering the cell cycle resulting in polyploidy. [88, 89] p53 can further influence the spindle checkpoint indirectly via the protein Survivin. Survivin belongs to a class of proteins termed inhibitors of apoptosis (IAP) that inhibit apoptosis by specifically inhibiting the function of Caspases. [89] In contrast to the other IAP family members Survivin exerts additional, evolutionary conserved functions in the G_2/M phase of the cell cycle. To exert these functions Survivin expression is usually cell cycle dependent [90] and p53 (via direct binding to the Survivin promoter or activation of p21 [91] and/or Rb (via E2F binding) regulated. Hence in response to stress signals during G_2/M phase p53 allows cells to enter apoptosis by inhibiting Survivin.

1.2.4 Inhibitors of CDKs

1.2.4.1 Natural inhibitors of CDKs

If DNA damage or other kinds of cellular stress occurs cell cycle arrest is induced to provide time for repair [92] or if repair is no longer an option apoptosis is induced. A major group of proteins namely cell cycle dependent kinase inhibitors (CDKIs) that are target genes of p53 and/or other regulatory factors, are essential mediators promoting cell cycle arrest or apoptosis during so called checkpoints.

They are divided into two families, the KIP/CIP family consisting of $p21^{CIP1/WAF\ 1}$, $p27^{KIP1}$, and $p57^{KIP2}$ and the INK4a family consisting of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. Members of the INK4a family mainly exert their function at the G_1/S checkpoint by binding to CDK4/6 whereas the CIP/KIP family displays broader target spectrum inhibiting CDK4, CDK2 and CDK1. [46]

Members of both families are frequently lost in human cancer. [93]

1.2.4.2 Pharmacologic CDK inhibitors

Loss of cellular CDKIs in cancers provided the idea to compensate it by synthetic compounds and provided the rational to develop pharmacological inhibitors of CDKs. [94] Some of those pharmacologic CDKIs namely Olomoucine, Olomoucine II and Roscovitine were used during my diploma thesis and will be explained in more detail later (see Medications).

1.3 Apoptosis

As already mentioned apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. [95] It is an active, programmed process that avoids inflammation and is characterized by nuclear and cytoplasmic condensation and the formation of apoptotic bodies. Those apoptotic bodies are taken up mainly by macrophages and degraded within their phagosome. [96]

In contrast to apoptosis necrosis is a passive, degenerative process induced due to unexpected and accidental cell damage. In case of necrosis the cell content is released uncontrolled into the surrounding tissue hence leading to inflammation. [97]

Current chemotherapeutic strategies mainly exert their function by triggering apoptosis in cancer cells hence detailed knowledge on both apoptotic pathways namely the extrinsic and the intrinsic pathway is of fundamental importance. In both pathways cysteine proteases, so called caspases, exert crucial functions. Most caspases are synthesised as inactive pro-caspases and require activation by another caspase upon which they can cleave next to aspartate residues and initiate (initiator caspases) or exert (effector caspases) apoptosis. [98]

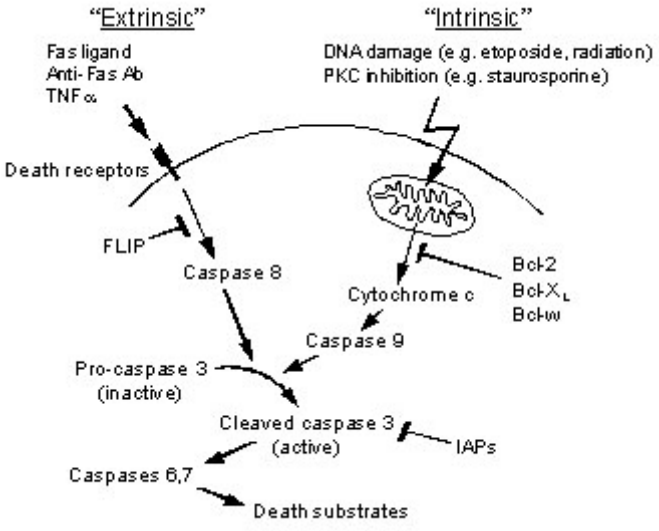

Figure 7. Apoptotic pathways (Graph by UCSD Health science)

1.3.1 Pathways of apoptosis

1.3.1.1 Extrinsic pathway

Upon stimulation by their corresponding ligands transmembran receptors such as death receptors of the tumor necrosis factor superfamily e.g. Fas/CD95 the Fas-associated death domain protein (FADD) [99] is recruited and in turn recruits the initiatior caspase 8 to form the death-inducing signal complex (DISC). By formation of this complex Caspase 8 is activated and is in turn able to cleave and thereby activate the effector caspase 3. [100] Targets of Caspase 3 include PARP, DNA-PKc, Mdm2, Lamin A/C.

1.3.1.2 Intrinsic pathway (=mitochondrial pathway)

The intrinsic pathway is initiated through the release of apoptotic factors such as cytochorm c, AIF (facilitates the DNA fragmentation), Smac/Diablo proteins (inhibit the inhibitor of apoptosis) by the mitochondrial intermembran space [101] and the activation of proapoptotic proteins such as Bax and Bid within the cell. [99] A complex composing of cytochrome c, ATP and Apaf-1 will thereafter recruit and activate caspase 9. Together those factors form the so termed apoptosome. The apoptosome is then able to activate the effector caspase 3 that will initiate degradation. [102, 103]

1.3.2 p53 in apoptosis

p53 in the intrinsic pathway of apoptosis

p53 is able to induce the intrinsic cascade of apoptosis by binding to the p53 RE (response element) in the promoters of several p53 targets. Targets of p53 relevant in apoptosis are pro-apoptotic proteins of the Bcl-2 family e.g. Bax [104] or proteins of the BH3-only family, [105] components of the apoptosis effector machinery e.g. Apaf-1 which is a co-activator of Caspase-9, or the effector Caspase-6.

p53 in the extrinsic pathway of apoptosis

Death receptors are also targets of p53 e.g. Fas/CD95 or DR5. [106]

1.3.3 Apoptosis in cancer

Both, the extrisic and the intrinsic pathway are often disrupted in cancer. The extrinsic pathway can be inhibited by increase in antiapoptotic molecules, by a derease or defective funciton in proapoptotic proteins and by downregulation, mutation or absence of surface receptors. The extrinsic pathway can be targeted by overexpression of Bcl-2 family proteins, decreased level or absence of Apaf-1 or mutations in the tumour suppressor p53. [107]

1.4 Hedgehog signalling

The hedgehog family of morphogenic proteins provide an evolutionary conserved pathway that is - next to other important signalling pathways such as Wnt or Notch signalling – one of the major regulators of embryonic development [119] and stem cell maintenance. [120] So far three mammalian hedgehog genes namely ‘Sonic hedgehog’, ‘Indian hedgehog’ and ‘Dessert hedgehog’ have been identified. Recently Gijs R. van den Brink and others postulated that a selection of tumours e.g. cancers of the gastrointestinal tract are hedgehog signalling dependent hence inhibitors of hedgehog could be potential new ways of targeting tumours even though the precise role of the hedgehog pathway in cancer is still unknown. [121] Because of this fact, the fact that hedgehog can influence Ras signalling [122] and the fact that hedgehog signalling can override p53 signals [120] one of the goals of my diploma thesis was to find out whether or not differences in hedgehog signalling are a reason why RECs cells expressing c-Ha-Ras isolated at different days of gestation behave differently.

Hedgehog signalling (**Fig. 8**) is initiated by Hedgehog ligand (Sonic Hedgehog, Desert Hedgehog and Indian Hedgehog) binding to its receptor Patched (Ptc) a 12-transmembran protein receptor in order to end Ptc mediated inhibition of a 7-transmembrane protein called Smoothend (Smo). Downstream of Smo is a multi-protein complex known as the Hedgehog signaling complex (HSC), which comprises the transcription factor Cubitus interruptus (Ci), the serine/threonine kinase Fused (Fu), the kinesin-like molecule Costal 2 (Cos2) and Supressor of fused (Sufu). Via Smo induced dissociation of this complex Ci is freed from Sufu repression and can activate its target genes. [123]

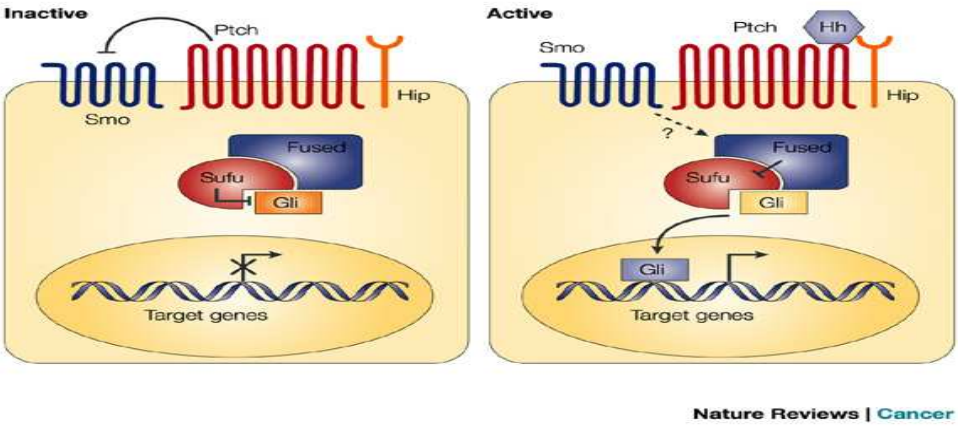

Figure 8 Hedgehog signalling

(Graph by: Marina Pasca di Magliano & Matthias Hebrok, nature rewiews 2003)

2. AIMS

The aims of this diploma thesis are:

4. Characterization of the action of Olomoucine II (OLO II), a new pharmacological CDK inhibitor on human healthy as well as cancer cells.
5. Assessment of the anti-proliferative and/or pro-apoptotic effects exerted by three closely related tri-substituted purines: Olomoucine (OLO), Roscovitine (ROSC) and Olomoucine II (OLO II) on human cancer cells (HL-60 promyelocytic leukaemia cells, HeLa cervical carcinoma cells), on human normal lung fibroblasts (MRC-5) and on immortalized (overexpression of temperature sensitive $p53^{135Val}$) as well as transformed (overexpression of $p53^{135Val}$ in combination with mutated c-Ha-Ras) rat fibroblasts.
6. Evaluation of consequences the loss of the G1/S checkpoint in cancer cells on the effectiveness of the treatment by CDK inhibitors.
7. Determination of the significance of the functional status of *TP53* for the susceptibility of cancer cells to therapy by CDK inhibitors.
8. Evaluation of the role of the expression and activity of c-Ha-Ras protein on the efficacy of the therapy by CDK inhibitors.
9. Comparison of the susceptibility of the rat cell clones generated from embryonic cells isolated from rat embryos of different age (13.5 or 15.5 gestation day) to treatment with CDK inhibitors.

This work is based on the results presented in the following papers:

Paper I:

Wesierska-Gadek J., Susanne B. Hajek, Bettina Sarg, Stefani Wandl, **Eva Walzi** Lindner H. 2008. Pleiotropic effects of selective CDK inhibitors on human normal and cancer cells'

Paper II:

Wesierska-Gadek J., Borza A., **Walzi E.,** Krystof V. , Maurer M., Komina O., Wandl S. 2009. Outcome of treatment of human HeLa cervix carcinoma cells with roscovitine strongly depends on the dosage and cell cycle status prior to the treatment. J. Cell. Biochem. (online published on January 23, 2009)

Paper III:

Wesierska-Gadek J., **Walzi E**., Schmid G. Functional status of p53 and Ras in tumor cells determined the susceptibility to treatment with pharmacological CDK inhibitors. (paper in preparation)

3. MATERIAL

3.1 Cell lines

All cell lines were cultivated till they reached a confluence of about 70 – 80 %. Except for the cell lines 189/111 and 173/1022 (see bottom) the cell culture was maintained without antibiotics.

3.1.1 HeLa S3

HeLa cells are the first continuously growing cancer cell line established more than 50 years ago. Back then the cancer sample was taken from a young black woman called Henrietta Lacks who suffered from glandular cancer of the cervix. In contrast to normal cervical cancer this tumour grew vicariously leading to the death of the patient 8 month after diagnosis. Within a few years after her death HeLa cell had been distribute in laboratories worldwide and are still of great importance in research fields such as cancer research. [108] One of the reasons why this form of cervical carcinoma is so aggressive is that it expresses the proteins E6 an E7 of integrated high risk human papilloma virus 18 (HPV-18). E6 and E7 display profound effects on the cell cycle of infected cells as E6 is able to bind p53 and target it for ubiquitin-mediated degradation and E7 is able to bind to hypophosporylated Rb protein leading to its destabilization and the disruption of the Rb-E2F complex. [109] Degradation/destabilization of those two leads to aberrant checkpoint control. [110] To sum things up, HeLa cells are both p53 and Rb negative.

HeLa cells were cultivated in RPMI medium containing 10% FCS at 37°C, 5% CO_2

3.1.2 MRC-5

MRC-5 cells are human wild-type fibroblasts derived from lung tissue of a 14-week-old male fetus by J.P. Jacobs in September of 1966. [111] MRC-5 cells are primary cells hence they have limited proliferative capacity (42 to 46 population doublings) before the onset of senescence. (ATCC) Further MRC-5 cells display a low mitotic index.

MRC 5 cells were cultivated in DMEM red medium containing 10% FCS at 37°C, 8% CO_2

3.1.3 HL 60

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, proliferates continuously in suspension culture and consists predominantly of promyelocytes. [112] Just like HeLa cells HL-60 cells exhibit a few genomic abnormalities like a 15-to 30fold genomic amplification of c-myc as compared with normal cells. [113, 114] A large body of physiological evidence shows that either upregulation or downregulation of the trancription factor c-myc activity has profound consequences on cell cycle progression [115] Further the N-ras proto-oncogenes has been reported to be mutated in 20% to 60% of samples of acute myelogenous leukemia [116] (JP Radich 1990)**.**

Just like mutations in c-myc constitutive active N-ras protein causes deregulation of the cell cycle. Furthermore the tumour supressor p53 is not expressed in HL-60 leading to further deregulation of the cell cycle and apoptosis. [117]

HL-60 cells were cultivated in RPMI medium containing 10% FCS at 37°C, 5% CO_2.

3.1.4 Rat fibroblast cell lines

Wesierska et al (1990) constructed primary Fisher rat embryo cells (RECs) containing temperature sensitive mutant mouse p53 or mutant mouse p53 in combination with mutated c-Ha-Ras. Overexpressed p53 does usually lead to cell cycle arrest or apoptosis thats why its not possible to study p53 regulatory functions under normal circumstances. Using a temperature sensitive mutant this problem can be circumvented and can provide important information about p53. Co-expression of mutant p53 with mutant c-Ha-Ras is of special interest as it allows us to investigate the effects of double mutants in a tumoursuppressor and a protoncogene. Used plasmids were pLTRp53cGval135; a chimera of mouse p53 cDNA and genomic DNA that encodes a temperature sensitive mutant protein with a substitution from alanine to valine at position 135; which integrates into the rat genome. The second plasmid is called PVEJB which encodes a mutated c-Ha-Ras gene and a Neomycin selective marker. [118]

The RECs were isolated from the head of rats at different time points namely 13.5 or 15.5 days gestation to evaluate potentially different influences of the microenvironment on cells of different age. The four established rat cell line were named 189/111 (isolated at day 13.5 gestation, containing mutant p53 + Ha-Ras), 173/1022 (isolated at day 15.5 days gestation,

containing p53 + Ha-Ras), 402/534 (isolated at day 13.5 days gestation, containing mutant p53) and 602/53 (isolated at day 15.5 days gestation, containing mutant p53).

Compared only by morphologic and ‘cell culture’ features the four cell lines and the wild-type primary rat fibroblast cell line 110/112 can already be distinguished (**Fig. 9**). 402/534 (young RECs, p53) and 602/534 (old RECs, p53) look very similar to wild-type fibroblasts hence no prominent differences can be detected under the light microscope whereas 189/111 (young RECs, p53, c-Ha-Ras) and 173/1022 (old RECs, p53, c-Ha-Ras) are way slimmer and longer than wild-type cells and the other two cell lines.

Further 189/111 and 173/1022 cell lines divide way faster than 110/112 cells or cells only containing temperature sensitive p53 instead of p53 + c-Ha-Ras. One more interesting features is that 189/111 and 173/1022 cells completely 'recover' form passaging after 18 hours but 402/534 and 602/534 cell need at least 24 hours to do so otherwise they show a strong increase in G1 cell cycle phase that disappears if the cells are allowed to 'settle' longer (data not shown).

Rat fibroblasts were cultivated in DMEM red medium containing 10% FCS at 37°C, 8% CO_2

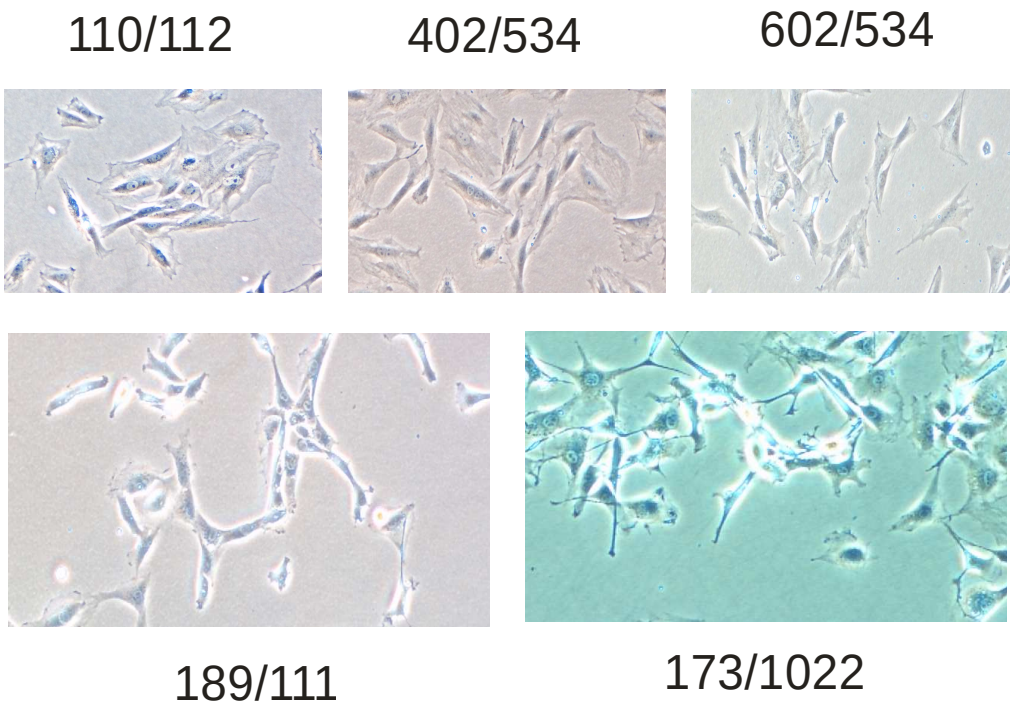

Figure 9. Morphologic features of REC cell lines. Light microscopic photography

3.2 Medications

All stocks of chemotherapeutics were sterilized by the use of a filter of 0.2 μm pore size.

Pharmacological CDK inhibitors

As CDKs are major components involved in all fundamental processes in the cell cycle they soon became promising therapeutic targets. Inhibition of CDK kinase activity has turned out to be a very efficient way to inhibit cancer cell cycle progression hence novel therapeutics often display similar effects as cellular CDKIs

3.2.1 Olomoucine (OLO)

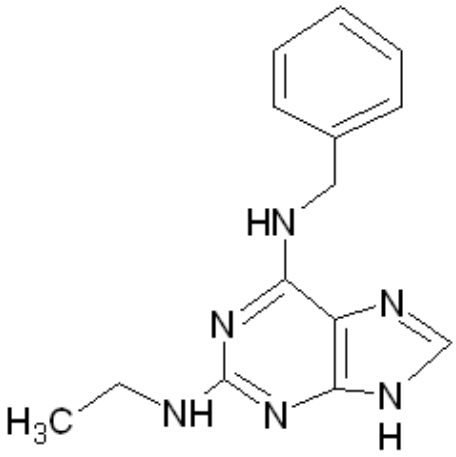

Figure 10. Molecular structure of OLO

Olomoucine, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine, is a purine derivate that inhibits cell cycle dependent kinases 1, 2, 5 [124] by competing for the ATP binding cleft in the catalytic site of the CDKs with ATP. [125] As ATP is required for CDKs to phosporylate their targets this leads to inhibiton of target phosphorylation.

The Olomoucine master mix (50mM) was solved in DMSO; stocks for cell treatment were solved in the accurate medium (e.g. RPMI or DMEM). DMSO in treated cell samples never exceeded a maximum concentration of 2%.

3.2.2 Olomoucine II (OLO II)

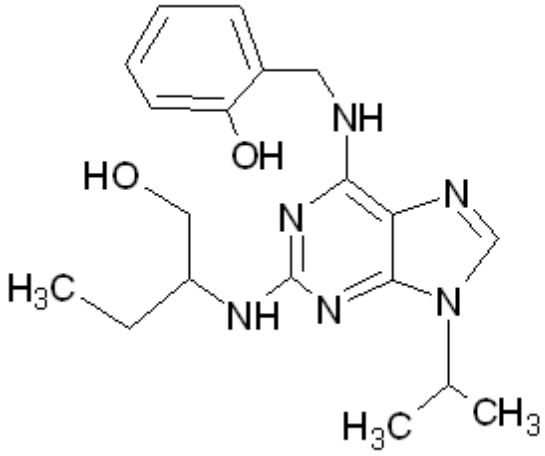

Figure 11. Molecular sturctur of OLO II

Olomoucine II, 6-[(2-hydroxybenzyl) amino]-2-{[1-(hydroxymethyl)propyl]amino}-9-isopropylpurine is a purine derivate just like Olomoucine. The only differences between Olomoucine and Olomoucine are a few structural changes that lead a higher inhibitory activity. [126] Apart from the main cell cycle-regulating kinases CDK1 and 2, Olomoucine II exerts specificity for CDK7 and CDK9, with important functions in the regulation of RNA transcription. OLO II was further reported to lead to activation of p53 hence accumulation of p21 and to inhibiton of Mdm2 ubiquitin ligase funcion. [127]

The Olomoucine II master mix (100mM) was solved in DMSO, stocks for cell treatment were solved in the accurate medium (e.g. RPMI or DMEM). DMSO in treated cell samples never exceeded a maximum concentration of 2%.

3.2.3 Roscovitine (ROSC)

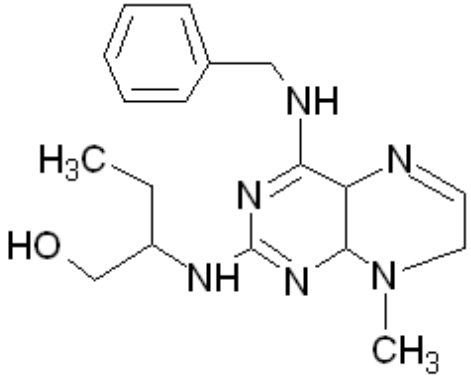

Figure 12. Moleculare structure of ROSC

Roscovitine (also named Seliciclib) is a 2,6,9-substituted olomoucine-related purine analog that preferentially inhibits CDK1, 2, 7 and 9. [128] It further induces stabilization, activation and accumulation of p53 in the nucleus via suppression of Mdm2 expression. [129]

The Roscovitine master mix (50mM) was solved in DMSO; stocks for cell treatment were solved in the accurate medium (e.g. RPMI or DMEM). DMSO in treated cell samples never exceeded a maximum concentration of 2%.

3.2.4 Cisplatin (CP)

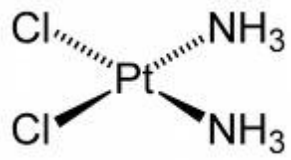

Figure 13. Molecular structure of CP

(by: wikimedia.org)

Cisplatin ((SP-4-2)-Diamminedichloroplatinum) is an alkylating agent that inhibits cell cycle progression cell cycle phase independent. It is one of the most widely used and most potent chemotherapy drugs. However, side effects in normal tissues and organs, notably nephrotoxicity in the kidneys, limit the use of cisplatin and related platinum-based therapeutics. [130]. The damaged DNA elicits DNA reapair mechanisms, which in turn activate apoptosis when repair proves impossible. [131]

A 1660µM stock of Cisplatin was provided by the company Platidima.

3.2.5 Etoposide (VP-16)

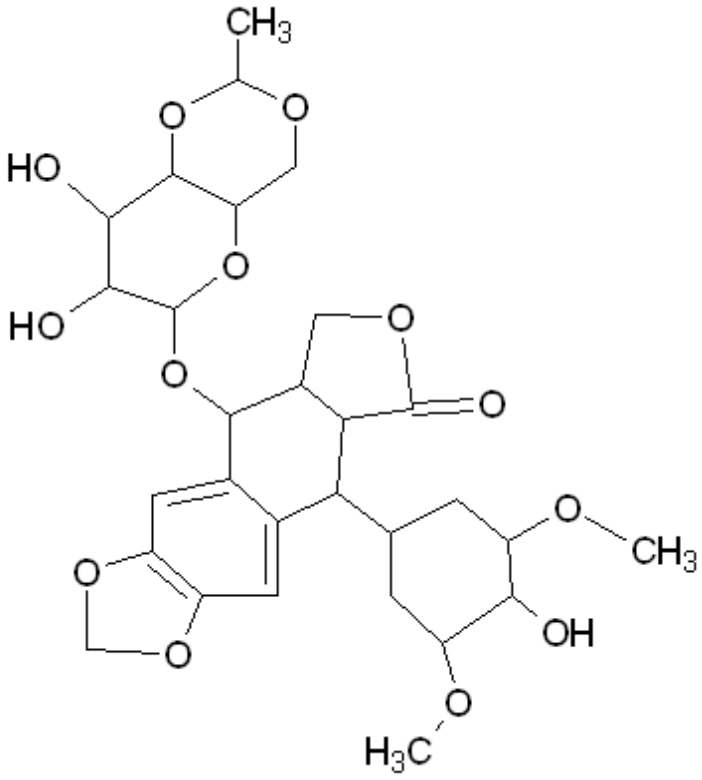

Figure 14. Molecular structure of VP-16

Epipodophyllotoxin derivatives, such as etoposide (VP16), constitute an important class of anticancer agents belonging to a class of plant alkaloids. They inhibit DNA synthesis by forming a complex with Topoisomerase II and DNA leading to protein-DNA cross links (so called cleavable complexes) and nicked DNA. [132] Accumulated breaks in DNA prevent entry into the mitotic phase of cell cycle and lead to cell death.

The Etoposide master mix (50mM) was solved in DMSO; stocks for cell treatment were solved in the accurate medium (e.g. RPMI or DMEM). DMSO in treated cell samples never exceeded a maximum concentration of 2%.

3.2.6 Nocodazole

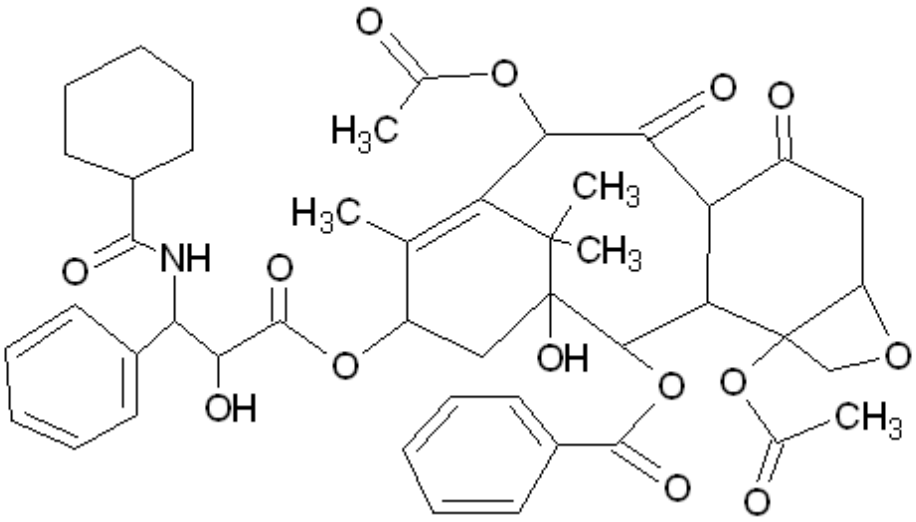

Figure 15. Molecular structure of NOCO

Nocodazole is a commonly used, synthetic, inhibitor of microtubul formation. [133] Due to lack of microtubul formation hence impaired mitototic spindle formation a reversibel block in the M phase (prometaphase) of the cell cycle is induced. [134]

The Nocodazole master mix (5mg/mL) was solved in DMSO, stocks for cell treatment were solved in the accurate medium (e.g. RPMI or DMEM). DMSO in treated cell samples never exceeded a maximum concentration of 2%

3.2.7 Cyclopamine

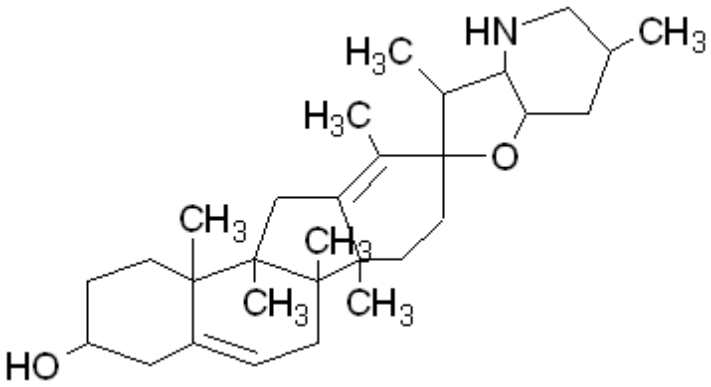

Figure 16 Molecular structure of Cyclopamine

Cyclopamine is a naturally occurring steroidal alkaloid that causes cyclopia (birth defect) by specifically blocking the sonic hedgehog pathway component Smoothened. [135]

The Cyclopamin master mix (5mM) was solved in DMSO, stocks for cell treatment were solved in the accurate medium (e.g. RPMI or DMEM). DMSO in treated cell samples never exeeded a maximum concentration of 2%

3.3 Cell culture material

RPMI-1640 Medium	Sigma
DMEM red Medium	Sigma
heat inactivated foetal calf serum (FCS)	PAA Laboratories GmbH
Difco Typsin250	BD
G418 (neomycin derivate) 50 mg/ml stock solution work concentration: 200 µg/ml	Roth
Cell culture dishes	Corning Inc.
Cell culture flasks	Corning Inc.
Cell Scraper	Sarstedt
Filter 0.2 µm (RC15, RC 25)	Sartorius
Incubator, Automatic Co_2 Incubator	New Brunswick

The cell culture was maintained without antibiotics except for the transformed rat cell lines 189/111 and 173/1022, were selection for the c-Ha-Ras containing plasmid on G418 is required.

3.4 Purchased Assays

CellTiter-Glo Luminescent Cell Viability Assay	Promega
Apo-ONE Homogeneous Caspase-3/7 Assay	Promega
CytoTox-ONE Homogeneous Membrane Integrity Assay	Promega

Multilabel-Multitask Plate Counter (Wallac 1420 VICTOR)

3.5 Buffers

10x PBS

Dulbecco's phosphate buffered saline (DPBS) without calcium chloride diluted in a ten-times smaller volume than recommended by the producer	Sigma

1x PBS

10x PBS diluted 1:10 with H_2O

1x PBS-Tween 20

10x PBS diluted 1:10 with H_2O

0.1 % (v/v) Tween 20	Sigma

10x Blotting buffer – per litre

144 g Glycin	Carl Roth GmbH/ Merk
30 g Tris	AppliChem
2 g SDS (sodium dodecyl sulphate)	Carl Roth GmbH
add ddH_2O	

1x Blotting buffer (pH 8.3)

100 ml 10x Transfer buffer	
200 ml Methanol	Carl Roth GmbH
700 ml ddH_2O	

10x Electrophoresis buffer - per litre

144 g Glycine	Carl Roth GmbH/ Merck
30 g Tris	AppliChem
10 g SDS (sodium dodecyl sulphate)	Carl Roth GmbH
add ddH_2O	

10x TBS - per litre

24.2 g Tris base	AppliChem
80 g NaCl	Merck
add ddH_2O	

RSB (reticulocytes suspension buffer) buffer

10 mM Tris/HCl pH 7.4	AppliChem/Carl Roth GmbH
10 mM NaCl	
1.5 mM $MgCl_2$	Roth

RIPA (radioimmuno precipitation assay buffer) buffer

50 mM Tris/HCl (pH 7.4)	AppliChem/ Carl Roth GmbH
500 mM NaCl	Merck
1 % (v/v) NP-40 (Nonidet P-40)	United States Biochemical Corp.
0.5 % (w/v) Na-Deoxycholic acid	Carl Roth GmbH
0.1 % (w/v) SDS (Sodium dodecyl sulphate)	CarlRoth GmbH

3.6 Solutions

Solutions for flow cytometric DNA measurement:

Stock solution for flow cytometric DNA measurement (pH 7.6)

20.4 mM Tri-Sodium citrate x 2 H_2O	Merck
0.6 % (v/v) NP-40 (Nonidet P-40)	UnitedStates Biochemical Corp.
9 mM Spermine tetrahydrochloride	Sigma
3 mM Tris	AppliChem

Solution A containing Trypsin

9 mg Difco Trypsin 250	BD
50 ml stock solution (pH 7.6)	

Solution B containing trypsin inhibitor and RNase A

150 mg Chicken Egg White (type II-0 = Trypsin inhibitor)	Sigma
30 mg Ribonuclease A (boiled before use for 3 minutes)	Sigma
50 ml stock solution (pH 7.6)	

Solution C containing propidium

4.9 mg Propidium iodide	Sigma
69.6 mg Spermin tetrahydrochloride	Sigma
10 ml stock solution (pH 7.6)	

20 mM Pefablock stock solution (5 µl/100 µl = 1 mM*)*

12 mg Pefa-Block (protease inhibitor)	Merck
50 ml ddH_2O	

0.1 M PMSF (Phenylmethylsulfonyl fluoride) stock solution

0.1 M PMSF	Calbiochem/Sigma

10 % APS (Ammonium persulphate)

1 g APS	Carl Roth GmbH
10 ml ddH_2O	

Carbonic Anhydrase I /BSA marker for protein gels (20 µl/lane)

20 µg/µl BSA (Bovine serum albumin)	Amresco
20 µg/µl Carbonic anhydrase I	Sigma
120 µl 2x SDS sample buffer stained/reduced	

20 % SDS (Sodium dodecyl sulfate)

20 g SDS	CarlRoth GmbH
100 ml ddH_2O	

SDS/NaN_3/Tris buffer for 2x SDS sample buffer (pH 6.8) - per 100 ml

6,006 g Tris	AppliChem
0.4 g SDS (Sodium dodecyl sulphate)	CarlRoth GmbH
0.01 g NaN_3	Sigma

2x SDS sample buffer non-stained/non-reduced - per 25 ml

0.5 g SDS (Sodium dodecyl sulfate)	CarlRoth GmbH
2.5 mg EDTA (Ethylenediaminetetraacetic acid)	Merck
5 mg NaN_3	Sigma
5 ml Glycerol	Amresco
1.25 ml SDS/NaN_3/Tris buffer for 2x SDS sample buffer (pH 6.8)	

2x SDS sample buffer stained/non-reduced - per 25 ml

0.5 g SDS (Sodium dodecyl sulphate)	CarlRoth GmbH
2.5 mg EDTA (Ethylenediaminetetraacetic acid)	Merck
5 mg NaN_3	Sigma
5 mg Bromphenol blue	United States Biochemical Corp.
5 ml Glycerol	Amresco
1.25 ml SDS/NaN_3/Tris buffer for 2x SDS sample buffer (pH 6.8)	

2x SDS sample buffer stained/reduced

1 ml 2x SDS sample buffer non-reduced	
20 µl 2.6M DTT (Dithiothreitol) stock solution	Sigma

Ponceau S

Ponceau-S working solution	
0.1 % (w/v) Ponceau-S (Certistain)	Merck
5 % (v/v) Acetic acid	Fluka Chemika

3.7 Other Chemicals

7-actinomycin A (7-AAD) solution	Sigma
Acrylamide/bis-Acrylamid 30%	Sigma
Aceton	Merck
BSA (bovine serum albumin)	PAA laboratories GmbH
DAPI (4'-6-diamidino-2-phenylindole)	Sigma
DMF (Dimethylformamid)	USB
DMSO (Dimethyl sulfoxide)	Sigma
ECL blot detection system reagent A	Amersham
ECL blot detection system reagent B	Amersham
Ethanol (70%)	Alkoholhandels Gmbh
Fluorescent mounting medium	Dako
Hoechst solution 33342	Invitrogen
JC-1 solution	Alexis Biochem.
$MgCl_2$	Roth
Milk powder	Fixmilch Instant
Natrium acid (NaN_3)	Sigma
NaF	Merck
NaOH	Merck/Roth
Na-Vanadat	Sigma
Paraformaldehyd	Merck
Protein Assay solution A	Bio Rad
Protein Assay solution B	Bio Rad
RNAse A (20 mg/mL)	Sigma
Sucrose	USB
TEMED (Tetramethylethylendiamin)	Roth

3.8 Antibodies

Primary antbodies:

M30 CytoDEATH, Fluorescein, mouse monoclonal antibody	Roche
mouse monoclonal anti-p53 (PAb 421) antibody	Oncogene
Ha-Ras antibody	DAKO

Seconsdary antibodies:

anti-mouse IgG secondary antibody, Cy2 labelled	Jackson ImmunoResearch Laboratories Inc.
anti-mouse IgG secondary antibody, Biotin labelled	Southern Biotechnology Associates, Inc.
Strepdavidin coupled tertiary antibody, Cy3 labelled	Sigma

4. METHODS

4.1 Seeding of cells

4.1.1 Seeding of cells growing as suspension culture

1. Thoroughly rinse the cell suspension culture from a bottle using a glass pipette
2. transfer medium, containing cells, into a Falcon tube
3. centrifuge at 200 g for 3 min at RT
4. discharge supernatant (old medium + dead cells)
5. resuspend cell pellet in fresh medium
6. transfer cells (amount depends on required cell density) to a new cell culture bottle filled with fresh medium (volume of medium depends on cell culture bottle size; small: 5 ml; medium: 13 ml; big: 20 ml)
7. number of cells in cell suspension is determined using a Bürker-Türk chamber
8. the resuspended cells are then diluted to a density of 5×10^4 per ml with medium
9. transfer cells of that density to Petri dishes (PDs), 96-well microtiterplates etc.
10. PDs or well microtiterplates are then incubated at 37°C for 24 hours (cells are allowed to rest)
11. after 24 hours cells can be exposed to drugs
12. further procedure as follows

4.1.2. Seeding of adherent cells

1. Remove medium form cell culture bottle
2. rinse bottle with 2 - 3 ml of Trypsin to remove remaining medium
3. add 2 - 3 ml of Trypsin to detach cell from cell culture bottle
4. incubate at RT or at 37°C until all cells become detached
5. inhibit Trypsin by adding an equal amount of medium supplemented with 10 % FCS
6. transfer the suspension to a Falcon tube
7. centrifuge at 200 g for 3 min at RT
8. proceed as listed in step 5 - 12 of the seeding procedure for suspension culture

4.2 Determination of the number of living cells

4.2.1 CellTiter-Glo Luminescent Cell Viability Assay

1. Check status of cell cultures (100 µl à 5 x 10^3 cells per well of 96 well plate) under the light microscope to evaluate the cell density, bacterial contaminations etc.
2. centrifuge plate at 200 g for 5 min at RT
3. add equal volume of LumiGLO reagent (substrate + buffer) to each well
4. mix gently
5. incubate for 20 min at 37°C to lyse cells
6. measure luminescence

4.2.2 Cell staining

4.2.2.1 Detection of cellular antigens by immunofluorescent staining

1. Check PD containing 2 ml à 5x10^4 in Ø 3cm PDs under the light microscope for contaminations etc
2. remove medium
3. wash trice (in the PD) with cold 1x PBS for 5 min
4. fixate cells using MeOH/Aceton (3:2) for 30 min at -20°C – take care not to let cells dry up (leave a bit of PBS behind before adding MeOH/Aceton)
5. wash trice with cold 1x PBS for 5 min
6. permeabilize cells by slowly adding 0.2 % Trition X-100 in 1x PBS
7. leave for 20 min
8. wash trice with cold 1x PBS for 5 min
9. block cells in 1x PBS containing 3 % BSA or 5 % milk powder for at least 1 hours at RT (better over night)
10. wash trice with cold 1x PBS for 5 min
11. add primary antibody (1:300 in T- PBS containing 3 % BSA or 5 % milk powder) and incubate for at least 2 hours at RT
12. wash 5 - 6 times with T- PBS for 10 min
13. incubate with fluorescent secondary antibody (1:500 in T- PBS containing 3 % BSA or 5 % milk powder) for at least 1 hours at RT

14. wash 5 - 6 times with T- PBS for 10 min
15. if required DAPI (in mounting medium see DAPI staining) can be added
16. use correct filter to check cells under the fluorescent microscope

4.2.2.2 Visualization of nuclei by DAPI staining

1. Check PD containing 2 ml à 5 x 10^4 in Ø 3cm PDs under the light microscope for contaminations etc
2. remove medium
3. wash trice (in the PD) with cold 1x PBS for 5 min
4. fixate cells using MeOH/Aceton (3:2) for 20 min at 4°C – take care not to let cells dry up (leave a bit of PBS behind before adding MeOH/Aceton)
5. wash trice with cold 1x PBS for 5min
6. permeabilize cells by slowly adding 0.2 % Trition X-100 in 1x PBS
7. leave for 20 min
8. wash trice with cold 1x PBS for 5 min
9. cover cells with DAPI solution:
    a. dilute stock solution (20 mg DAPI in 2 ml DMF) 1:10 in 1x PBS
    b. dilute the dilution 1:25 in mounting medium
10. carefully add ~500 µl of 1x PBS to avoid drying
11. use correct filter to check cells under the fluorescent microscope

4.2.2.3 Visualization of chromatin structure by Hoechst staining

1. Check PD containing 2 ml à 5 x 10^4 in Ø 3cm PDs under the light microscope for contaminations etc
2. remove medium
3. wash trice (in the PD) with cold 1x PBS for 5 min
4. fixate cells using 4 % Paraformaldehyd in 1x PBS (pH 7.4) for 30 min at 4°C
5. wash trice with cold 1xPBS for 5 min
6. dry PD
7. add 2 ml of Hoechst solution (2 µg/ml) dissolved in FACS solution B (see material)
8. incubate PD for 30 min at 4°C in the dark
9. wash with ddH_2O
10. use correct filter to check cells under the fluorescent microscope

4.3 Determination of the distribution of cells in distinct cell cycle phases

4.3.1 DNA staining by FACS analysis

1. Check status of cell cultures (4 ml à 5 x 10^4 in Ø 6cm PDs) under the light microscope to evaluate the cell density, bacterial contaminations etc.
2. transfer medium into a Falcon tube
3. wash Petri dish with 1x PBS to remove medium and add the liquid to the Falcon tube
4. detach adherent cells by adding Trypsin (1 - 2 ml) and incubate at RT or at 37°C until all cells become detached (not necessary for suspension cutlures)
5. inhibit Trypsin by adding an equal volume of FCS supplemented medium (not necessary for suspension cultures)
6. centrifuge at 900 g for 3min at 4°C
7. wash cells twice with 1x PBS
8. resuspend cell pellet in approximately 100 µl of 1x PBS (depending on pellet size)
9. transfer resuspended cells into a FACS tube
10. add 75 µl of the FACS solution A
11. incubate for 10min at RT
12. add 63 µl of the FACS solution B
13. incubate for 10min at RT
14. add 63 µl of the FACS solution C (Propidium iodide staining solution)
15. incubate for at least 30 min at 4°C
16. measure the fluorescence (Ex/Em: 530/615nm)

4.3.2 Fixation of cells with ethanol (in case cells can not be measured right away)

Fixation:

1. Check cells (4 ml à 5 x 10^4 in Ø 6cm PDs) under the light microscope for contaminations etc.
2. pour medium into a Falcon tube
3. wash Petri dish with 1x PBS to remove medium and add the liquid to the Falcon tube
4. detach adherent cells by adding 1 - 2 ml Trypsin and leave until cells are detached (not necessary for non-adherent cells)

5. inhibit Trypsin by adding an equal amount of medium (not necessary for non-adherent cells)
6. centrifuge at 900 g for 3min at 4°C
7. wash with 5 ml 1x PBS
8. centrifuge at 200 g for 6 min at 4°C
9. resuspend the pellet in 500 µl 1x PBS
10. Transfer the suspension to a Falcon tube containing 4.5 ml ice cold 70 % ethanol
11. vortex thoroughly
12. store at -20°C until measurement

Staining procedure:

1. Spin down ethanol-fixed cells at 200 g for 5 min at 4°C
2. Discard ethanol
3. Suspend the pellet in 5 ml 1x PBS and wait 60 seconds
4. Centrifuge at 200 g for 5 min
5. Resuspend fixed cells in 500 µl of the following staining solution:
6.

   9.9 ml 1xPBS

   0.1 ml RNAse A (20 mg/ml)

   0.1ml Triton X-100

   200 µl PI (1 mg/1 ml H_2O)
7. Measure the fluorescence (Ex/Em: 530/615nm)

4.4 Determination of programmed cell death

4.4.1 Apo-ONE Homogeneous Caspase-3/7 Assay

1. Check cells (100 µl à 5 x 10^3 per well of 96 well plate) under the light microscope for contaminations etc.
2. centrifuge plate at 200 g for 5 min at RT
3. transfer 50 µl of supernatant from each well to a black microtitor plate
4. add APO-ONE reagent 1:1 to supernatant and cells of drug treated samples, untreated control sample and blank (APO-ONE reagent: APO-ONE substrate + buffer 1:100)
5. mix gently
6. incubate 30 min - 18 hours

7. measure fluorescence over each well at 499/521nm

4.4.2 CytoTox-ONE Homogeneous Membrane Integrity Assay

1. Check cells (100 µl à 5 x 10^3 per well of 96 well plate) under the light microscope for contaminations etc.
2. centrifuge plate at 200 g for 5 min at RT
3. add 2 µl of lysis solution to 100 µl of untreated control cells (pos. control)
4. transfer 50 µl of supernatant from each well to a black microtitor plate
5. add CytoTox reagent 1:1 to supernatant and cells of drug treated samples, untreated control sample positive control and blank
6. mix gently
7. incubate 10 min
8. add stop solution
9. measure fluorescence of each well at 560/590nm

4.4.3 Determination of the integrity of plasma membrane by dye exclusion test using 7-AAD as a substrate

- Check cells (4 ml à 5 x 10^4 in Ø 10cm PDs) under the light microscope for contaminations etc.
- transfer medium into a Falcon tube
- wash Petri dish with 1x PBS to remove medium and add the liquid to the Falcon tube
- detach adherent cells by adding 1 - 2 ml Trypsin and leave until cells are detached (not necessary for non-adherent cells)
- inhibit Trypsin by adding an equal amount of medium (not necessary for non-adherent cells)
- centrifuge at 900 g for 3 min at 4°C
- wash twice with 1x PBS
- resuspend pellet in approximately 500 µl of 1x PBS (depends on pellet size)
- transfer resuspended pellet into a FACS tube
- add 5 µl 7 AAD
- incubate in the dark for 20 min at RT

- measure the fluorescence (Ex/Em: 555/655nm)

4.4.4 Determination of the potential of mitochondrial membrane using JC-1 dye as a substrate

1. Check cells (4 ml à 5 x 10^4 in Ø 6cm PDs) under the light microscope for contaminations etc.
2. transfer medium into a Falcon tube
3. wash Petri dish with warm (37°C) 1x PBS to remove medium and add the liquid to the Falcon tube
4. detach adherent cells by adding 1 - 2 ml Trypsin and leave until cells are detached (not necessary for non-adherent cells)
5. inhibit Trypsin by adding an equal amount of medium (not necessary for non-adherent cells)
6. centrifuge at 900 g for 3 min at ~25°C
7. wash with warm 1x PBS twice
8. add 10 µl JC-1 solution (10 µl warm JC-1 stock solution [5 mg in 766 µl DMSO]+ 90 µl warm DMSO)
9. add 490 µl of warm 1x PBS and resuspend cells
10. incubate in the dark at 37°C for 20 min
11. wash with warm 1x PBS 3 times
12. resuspend pellet in ~200 µl of PBS (depends on pellet size)
13. measure immediately fluorescence at two channels (green: Ex/Em 510/527nm; red: Ex/Em 585/590nm)

4.4.5 Flow cytometric determination of the caspase-3 mediated cleavage of cytokeratin 18 by staining using FITC-coupled M30CytoDEATH monoclonal antibody

1. Check cells (4 mL à 5 x 10^4 in Ø 6cm PDs) under the light microscope for contaminations etc.
2. transfer medium into a Falcon tube
3. wash Petri dish with 1x PBS to remove medium and add liquid to Falcon tube
4. detach adherent cells by adding 1 - 2 ml Trypsin and leave until cells are detached (not necessary for non-adherent cells)

5. inhibit Trypsin by adding an equal amount of medium (not necessary for non-adherent cells)
6. centrifuge at 900 g for 3 min at4°C
7. wash with 1x PBS twice
8. Resuspend the pellet in 500 µl 1x PBS
9. add slowly 5 ml (10 fold amount of PBS) ice cold 70 % ethanol
10. vortex thoroughly
11. store at -20°C for maximal 30 min
12. Centrifuge 5 min, 200 g
13. Wash twice with 1x PBS + Tween
14. Resuspended in PBS sample can be stored at 4°C otherwise:
15. Centrifuge at 200 g for5 min at4°C
16. Resuspend the pellet in 100µL 1x PBS-T containing 1 % BSA
17. leave at 4°C for at least 1 hour (better over night)
18. Centrifuge at 200 g for 5 min at 4°C
19. Add 300 µl of FITC-coupled M30CytoDEATH antibody (1:300 in 1x PBS-T) and leave for at least 2 hours on RT
20. Centrifuge at 200 g for 5 min at 4°C
21. wash twice with 1x PBS-T
22. measure fluorescence generated by FITC-coupled M30-CytoDEATH antibody in cells undergoing apoptosis

4.5 Cell lysis and preparation of whole cell lysates (WCLs)

4.5.1 Lysis

1. Check cells (12 ml à 5 x 10^4 in Ø 10cm PDs) under the light microscope for contaminations etc.
2. pour medium into a Falcon tube
3. wash Petri dish with 1x PBS to remove medium and add the liquid to the Falcon tube
4. detach adherent cells by adding 1 - 2 ml Trypsin and leave until cells are detached (not necessary for non-adherent cells)
5. inhibit Trypsin by adding an equal amount of medium (not necessary for non-adherent cells)
6. centrifuge at 900 g for 3min at 4°C

7. wash with 1x PBS trice
8. resuspend pellet in approximately 1000 µl of 1x PBS
9. transfer resuspended pellet into a Eppendorf tube
10. centrifuge for 3 min at 1000 g
11. remove supernatant
12. add ~100 µl of PIPA lysis buffer (RIPA buffer + 1:100 µl PMSF + 1:500 µl PEFA + 1:1000 µl Na-Vanadat + 1:1000 µM NaF) and resuspend
13. put on ice for 30 min
14. sonificate cells for 3 times 10 sec
15. keep on ice

4.6 Cell fractionation and isolation of nuclei

1. Check cells (12 ml à 5 x 10^4 in Ø 10cm PDs) under the light microscope for contaminations etc
2. yield cells by scrapping
3. wash trice with cold 1x PBS
4. resuspend the pellet in ice-cold low salt buffer (RSB buffer) (see materials) → use about ten-fold volume of the pellet size
5. keep on ice for 15 min
6. slowly add non-ionic and ionic detergents (to a final concentration of 0.25 % NP-40, and of 0.15 % NaDoc, respectively), use a 10 % stock solution
7. vortex thoroughly
8. incubate on ice for at least 5 minutes
9. potter 10 - 20 times/centrifuge at 1000 g for 10 min at 4°C
10. take off the supernatant (cytosol) and store at -20°C
11. resuspend the pellet (crude nuclei) in 500 - 100 µl of RSB buffer (depending on pellet size)
12. prepare a Falcon tube containing around 4 ml of 0.25 M sucrose/10 mM $MgCl_2$ /carefully add the resuspended pellet on top of it→ make sure not to mix the pellet with the surose/$MgCl_2$ so that a gradient can establish
13. centrifuge at 4000 g for 20 min at 4°C

14. dissolve the pellet in SDS sample buffer non-stained, non-reduced (small pellet) or RIPA lysis buffer containing PEFA block, PMSF, Sodium-Vanadat and Sodium fluoride (big pellet) and store at -20°C

4.7 Analysis of protein expression by immunoblotting

4.7.1 Determination of protein concentration

1. Centrifuge Eppendorf tube at 5000 g for 4 min at RT
2. add 5 µl of lysed cells to 15 µl of H_2O dest.
3. add 100 µl Bio Rad Protein Assay solution A
4. add 800 µl Bio Rad Protein Assay solution B
5. wait 15 min
6. measure samples and protein standards (BSA) at 750 nm

4.7.1.1 BSA Standard

Master Mix: 0.05 g BSA in 1ml (=50µg / µl)
Solution I: 1:25 diluted master mix 10 µl + 240 µlH2O
Solution II: 1:10 diluted solution I 10 µl + 90 µlH2O

´4.7.1.1.1 Standard curve

[BSA] [µg]	Vol. H_2O [µl]	Vol. solvent [µl] (RIPA solution)	Vol. BSA-solution [µl]
0	15	5	---
0	15	5	---
1	10	5	sol. II 5,0
2	5	5	sol. II 10,0
3	---	5	sol. II 15,0
3	---	5	sol. II 15,0
5	12.5	5	sol.I 2.5
7	11.5	5	sol.I 3.5
7	11.5	5	sol.I 3.5
10	10	5	sol.I 5.0
15	7.5	5	sol.I 7.5
20	5	5	sol.I 10.0
20	5	5	sol.I 10.0
25	2.5	5	sol.I 12.5
sample	15	5 µl original sample	---

Table 1. BSA standard kurve

4.7.2 SDS-PAGE

1. Cast a SDS polyacrylamide/bis-Acrylamide gel (acrylamide/bis-acrylamide concentration depends on the molecular weight range that has to be analysed):
   - 40 % acrylamide/Bis-acrylamide (ratio 40:1)
   - 20 % SDS
   - 10 % APS
   - TEMED
   - 2 M Tris/HCl pH 8.7 (for resolving gel) or pH 6.8 (for stacking gel)
   - H_2O bidest.

Resolving gels	**8 %**	**10 %**	**12 %**	**15 %**	**Stacking Gel**	**For all gels**
30 % Acrylamide	2300 µl	2900 µl	3480 µl	4350 µl	**30 % Acrylamide**	320 µl
2M TRIS/HCL pH 8.7	1650 µl	1650 µl	1650 µl	1650 µl	**1M TRIS/HCL pH 6.8**	300 µl
SDS 20 %	44 µl	44 µl	44 µl	44 µl	**SDS 20 %**	12 µl
APS 10 %	39.6 µl	39.6 µl	39.6 µl	39.6 µl	**APS 10 %**	12 µl
TEMED	6.6 µl	6.6 µl	6.6 µl	6.6 µl	**TEMED**	2.4 µl
ddH_2O	4759.8 µl	4160 µl	3579.8 µl	2710 µl	**ddH_2O**	1753.6 µl
Total	8.8 ml	8.8 ml	8.8 ml	8.8 ml	**Total**	2.4 ml

Table 2. SDS polyacrylamide/bis-Acrylamide gel

2. mix 30 µg sample + x µl 2x sample buffer stained, reduced - see material - of a total volume of 25 µl)
3. heat to 95°C for 5 min
4. store on ice for 5 min
5. spin down sample
6. apply to slab gel
7. run gel electrophoresis at 130 volt using a Novex-X-cell II apparatus filed with 1x electrophoresis buffer)

4.7.3 Protein transfer by electro-blotting

1. put gel on 2 slices of Whatman Paper
2. activate PVDF membrane in MeOH (10 sec)
3. put membrane on top of gel
4. put 2 slices of Whatman Paper on top of membrane
5. remove air bubbles
6. cover membrane/gel/Whatman Paper with blotting buffer soaked sponges

7. transfer into blotting chamber filled with blotting buffer
8. blot for 1h (120 V) or o/n (30 V)

4.7.4 Ponceau S staining

- Activate membrane in MeOH
- dye with Ponceau S (0.1 %(w/v) Ponceau S in 5 %(v/v) acetic acid for 5 - 10 min
- wash out Ponceau S with H_2O or MeOH

4.7.5 Membrane blocking to avoid unspecific binding

Incubate in:

~10 ml of 2.5 g milk in 50 ml 1x PBS + 0.1 % Tween 20 (non-phospho specific antibodies) or

~10 ml of 1.2 g BSA in 40 ml 1x TBS + 0.1 % Tween 20 (phospho specific antibodies)

Discard the saturating solution

4.7.6 Incubation with primary antibodies

1. Add primary antibody (1:1000 dilution in BSA / 1x TBS + Tween 20 or milk / 1x PBS + Tween 20) and incubate for 1 - 2 hours at RT or o/n at 4°C
2. wash at least 3 times 10 min with 1x TBS + Tween 20
3. incubate 1 - 2 hours with secondary antibody (1: 5 000 or 1:10 000; depending on the features of primary antibody used)
4. wash at least 3 times 10 min with 1x TBS + Tween 20
5. discard the solution

4.7.7 Detection of the immune complexes

1. Mix reagent A (1000 µl) with reagent B (25 µl) from ECL+ Western blot detection kit
2. load on membrane

3. incubate for 3 - 5 min
4. discard the solution and cover the membrane with Sarap wran
5. put a film on top of the membrane (in darkness)
6. keep membrane + film in the dark and incubate for required time (depends on antibody)
7. develop film

4.7.8 Stripping of membranes

1. Activate membrane in MeOH
2. wash membrane in ddH_2O for 5 min
3. transfer the membrane to 0.2 M NaOH (e.g. 0.5 ml NaOH in 9.5 ml dH_2O) and leave 5 min
4. wash membrane in ddH_2O for 5 min
5. restart with blocking (see blocking)

5. RESULTS

5.1 Anti-proliferative action of OLO and OLO II on human HL-60 leukaemia cells

Short- and long-term effects of OLO and OLO II on the number of living human HL-60 cells

The anti-proliferative effects of OLO and OLO II on human HL-60 leukaemia cells were assessed using 'CellTiter-Glo Luminescent Cell Viability assay'. As previously described in the section Material and Methods, this assay is based on the measurement of the concentration of adenosine triphosphate (ATP), an attribute of metabolically active cells, which allows the determination of the number of viable cells within the broad range. This one-step reagent contains firefly luciferase and luminol, its substrate. However, in the absence of ATP the reaction can not occur. Cells can be used as a source of ATP provided that the major components of the reaction encounter the nucleotide. Therefore, the reagent lyses cells and thereby releases ATP. This starts the reaction resulting in the generation of luminescence. The intensity of the generated light strongly depends on the encountered ATP concentration and in consequence on the number of living cells. Hence when compared to untreated control cells, the reduction of the ATP level in drug treated samples directly reflects the decrease of cell number of viable cells.

HL-60 cells were treated with the tested CDK inhibitors (OLO, OLO II) for 24 hours at the indicated concentrations and the cellular ATP level was determined immediately after treatment or after the medium was changed (MC) and cells were cultivated in a drug-free medium for further 48 hours. From the dose-response curve the so called half maximum inhibitory concentration (IC_{50}) for each drug and each condition was calculated. The IC_{50} indicates the concentration of a drug required to reduce the cell number by 50 % as compared to the corresponding control.

Low doses of OLO (up to 50 µM) do not lead to a significant reduction in cell number (see **Fig. 17a**). On the other hand high OLO concentrations (75 µM – 150 µM) - especially the highest dose (150 µM) – exert strong anti-proliferative effects on HL-60 cells. As indicated in Fig. 17a the IC_{50} for OLO was 104.4 µM for 24 hour treatment. After exposure of HL-60 leukaemia cells to 150 µM OLO for 24 hours the cell number was almost completely eliminated.

We further tried to determine so called 'long term effects' of OLO. For this purpose the culture medium was changed after treatment of HL-60 cells for 24h and cells were subsequently post-incubated in fresh, drug-free medium for further 48h. As shown in figure 17a, the IC_{50} value for HL-60 cells maintained under these conditions decreased by approximately 40 % (65.5 µM) thereby indicating that the consequence of CDK inhibition persisted even after two days in the absence of the drug.

As its name indicates, OLO II is the successor of the conventional OLO and just like OLO it has already displayed promising therapeutic effects on various cancer cell lines e. g. human MCF-7 breast cancer cells. In my diploma thesis we tried to elucidate whether OLO II would exert comparable effects on HL-60 cell proliferation or maybe even surpass those effects. As shown in figure 17b OLO II inhibited the proliferation of HL-60 cells at much lower concentration; its IC_{50} was 2.81 µM for 24 hour treatment and 1.84 µM when cell were subsequently post-incubated in a drug-free medium. The cell number was almost completely diminished after treatment with 5 µM OLO for 24 hours (only around 10 % of the original cell number remained).

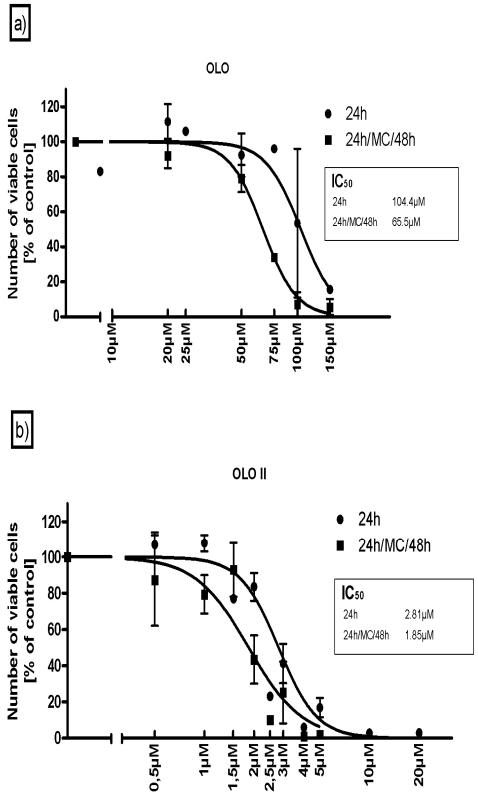

Figure 17. Reduction of the number of living HL-60 cells after exposure to increasing concentrations of OLO and OLO II

MC = medium change, IC_{50} = half maximum inhibitory concentration

Above results indicate that OLO II inhibited proliferation of HL-60 cells at approximately 40-fold lower doses.

The statistical significance of the results obtained for both agents was evaluated by appropriate tests (**Fig. 18a and b**).

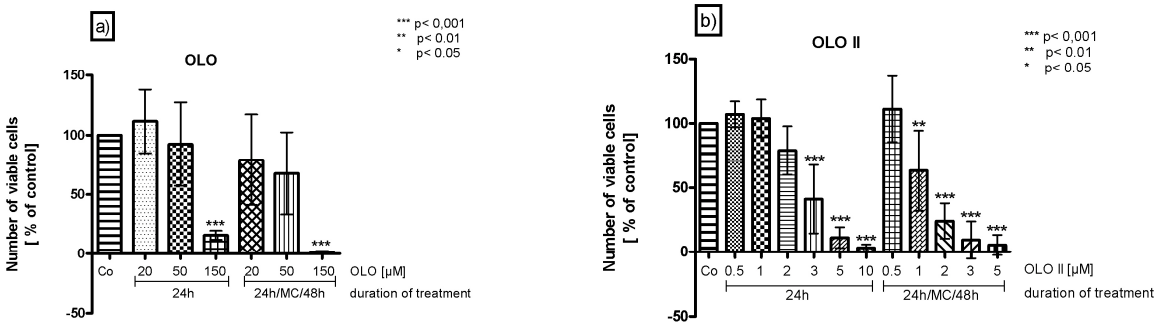

Figure 18. Significance of the action of OLO or OLO II on the number of HL-60 cells

Following statistic tests were used: One-way-ANOVA, Dunnett post test

The observation that both tested CDK inhibitors reduced the number of viable HL-60 cells does not give any information on the mechanisms of the observed effect. This diminution of cell number could be the result of cell cycle arrest induced by certain anti-cancer drugs or it could alternatively be due to direct cell killing. As both OLO and OLO II are known to act as pharmacological inhibitors of cyclin dependent kinase we examined the effect of OLO and OLO II on the cell cycle progression. Untreated cells and cells exposed to these drugs were collected, cells were stained with propidium iodide according to a well established procedure and the DNA concentration in single cells was determined by flow cytometry.

OLO and OLO II induce a weak cell cycle arrest in HL-60 cells

HL-60 cells were treated with 20, 50 and 150 µM of OLO for 24 hours. Cells were processed for propidium iodide staining immediately after treatment or additionally after post-incubation in a drug-free medium for further 24 or 48 hours. After continuous treatment for 24 hours no visible effects on the cell cycle progression could be detected at any drug concentration used except for 20 µM OLO. This apparently strong impact of OLO at such a low dose (20 µM) has to be confirmed in additional experiments since the values between distinct experiments strongly varied.

Interestingly it became apparent that treatment of HL-60 cell with OLO at a final concentration of 150 µM OLO resulted in an accumulation of hypoploid cells indicating that cells died by apoptosis.

Treatment with OLO seem to have long term effects

The size of the hypoploid cell population decreased during post-incubation period in a time-dependent manner. Compared to our results obtained with cells treated for 24 hours the pattern of cell cycle distribution changed after post-incubation of OLO-treated cells for 24 hours in drug-free medium. In this case a marked increase in G_2 cell cycle phase could be detected (**Fig. 19**). The increase of the frequency of the G_2 cell population was transient and disappeared after post-incubation for further 24 hours. Therefore, it seems that HL-60 cells are able to recover fast from OLO-induced cell cycle arrest.

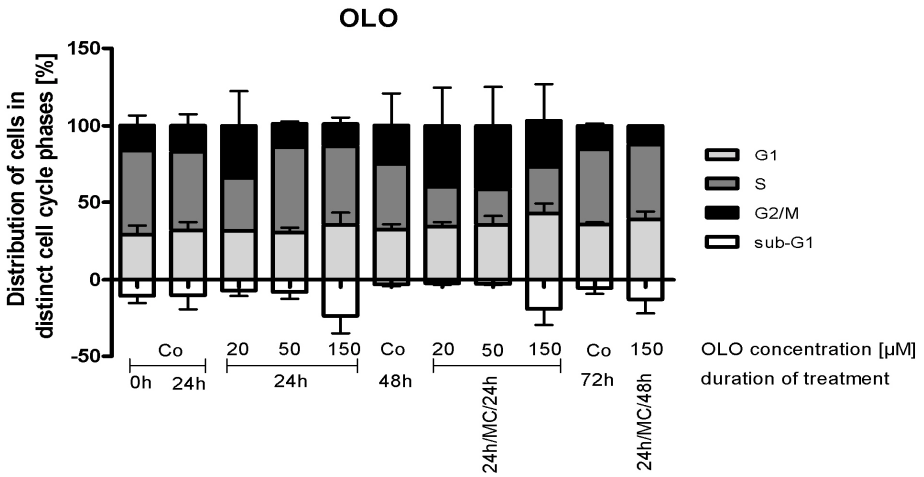

Figure 19. Effect of treatment with OLO on the distribution of HL-60 cells in distinct cell cycle phases

Drug concentration in µM

In the next step similar experiments were performed with OLO II, a second tested CDK inhibitor. HL-60 cells we treated with OLO II at concentrations ranging from 1 µM up to 20 µM for 24 hours. OLO II at low doses (1 and 2 µM) didn't induce cell cycle arrest (**Fig. 20**).

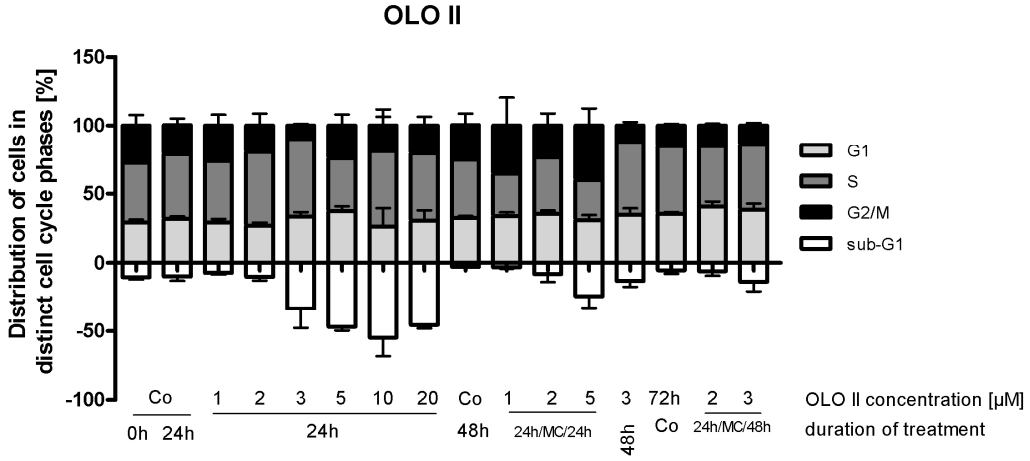

Figure 20. Effect of treatment with OLO II on the distribution of HL-60 cells in distinct cell cycle phases

Drug concentrations in µM

Higher doses (in our case 3, 5, 10 and 20 µM) led to a slight decrease of G_1 phase and concomitantly to an increase in G_2 phase of the cell cycle. This effect became especially apparent at concentrations of 10 µM OLO II. The same trend was observed after continuous treatment for 48 hours with 3 µM OLO II (**Fig. 21**). It is obvious that OLO II didn't diminish the ratio of S-phase cells.

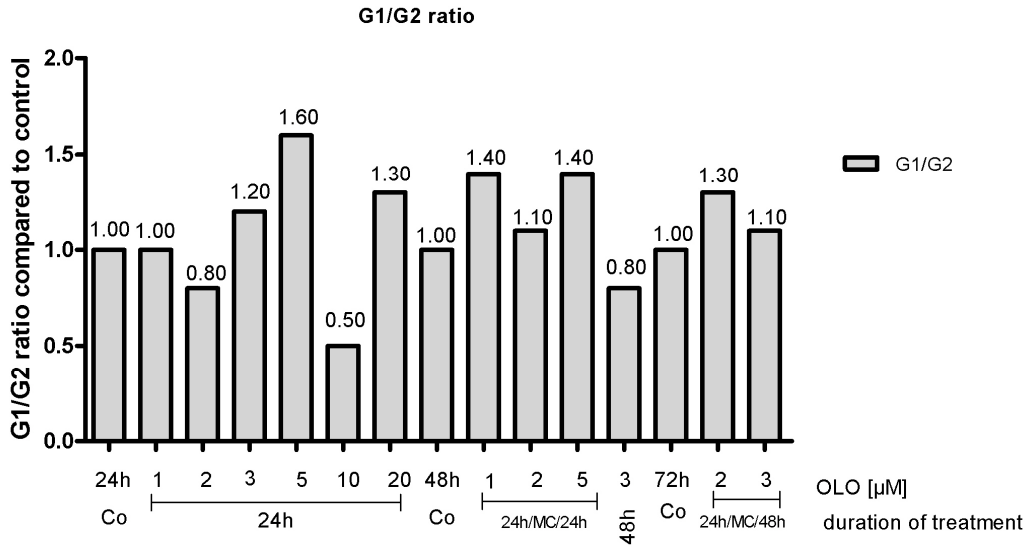

Figure 21. Comparison of G_1/G_2 ratio of HL-60 cells after treatment with OLO II.

Values were normalized against a corresponding control.

OLO II was previously shown to specifically inhibit CDK2 (responsible for G_1-S progression) and to activate p53 hence the cell cycle inhibitor p21 in a variety of cell lines. In HL-60 cells it seems realistic that OLO II exerts the same effect on CDK2 but that cell cycle progression is still possible due to lack of p53 until cells arrest in G_2 phase due to p53 independent mechanisms.

Exposure of human HL-60 cells to OLO II for 24 hours resulted in a marked accumulation of sub-G_1 cells. This effect was dose-dependent within the range from 2 µM to 10 µM. The highest frequency of hypoploid cells (up to around 50 % of the gated diploid cells) was observed after treatment with 10 µM of OLO II. (**Fig. 20**) At 20 µM the size of population of sub-G_1 cells decreased (**Fig. 20**).

Again, it was of great interest to determine how long the OLO II-induced changes of the distribution in the cell cycle phases may persist. Therefore, HL-60 cells treated with increasing concentrations of OLO II (1, 2, 3 and 5 µM) for 24 hours were post-incubated in drug-free medium for further 24 or 48 hours. Interestingly OLO II was still able to induce apoptosis in a concentration-dependent fashion after medium change and the cell cycle arrest became even more apparent after cultivation in a drug-free medium for 24 hours. In

comparison we were unable to show cell cycle arrest in cells treated with 2 or 3 µM OLO II for 24 hours that were subsequently transferred to a drug-free medium for 48 hours. Apoptotic cells were still detectable. Thus, it appears that HL-60 cells are able to rapidly recover from OLO II induces cell cycle arrest and that OLO II strongly exerts its main function on human leukaemia cells by other means – one of them probably being the induction of apoptosis.

OLO and OLO II induce caspases-dependent apoptosis in HL-60 cells

The accumulation of hypoploid HL-60 cells upon treatment with both studied CDK inhibitors indicated that the drugs trigger them to apoptosis. To ensure that accumulation of hypoploid HL-60 cells is attributable to the induction of apoptosis, we performed several methods and assays to determine whether OLO and/or OLO II induce apoptosis and to discriminate apoptosis from necrosis. For comparison, experiments were performed with two anti-cancer drugs frequently used in the clinical routine namely: cisplatin (CP) and etoposide (VP-16). Unlike OLO and OLO II, both, CP and VP-16, are strongly cytotoxic, generate free radicals and induce severe DNA damage.

As previously done, we treated HL-60 cells with drugs (OLO, OLO II, CP, VP-16) at different concentrations for 24 hours. After 24 hours APO-ONE assay was performed as described in the section “Methods”. The APO-ONE assay is designed to detect the activity of the two Caspases 3 and 7 that are effectors of the extrinsic and intrinsic pathway of apoptosis. APO-ONE reagent provides a pro-fluorescent substrate (rhodamine 110) for caspases and lyses/permeabilize cells. In the presence of activated Caspase 3/7, rhodamine 110-coupled substrate encompassing DEVD tetrapeptide motif is cleaved by the apoptotic proteases and leaving groups become fluorescent. The intensity of the generated fluorescence is directly proportional to the caspase 3/7 activity present in the sample. In a parallel assay the number of viable cells is determined by a cell viability assay e.g. the previously used ‘CellTiter-Glo Luminescent Cell Viability assay’. Quantification is achieved after dividing the measured fluorescence (relative fluorescence unit [RFU]) by the number of viable cells. Finally, values obtained for distinct samples are normalized against those determined in untreated controls (% of control cells).

The activity of caspase 3/7 was determined separately in the culture supernatant and in cells. Considering the fact that a difference in the amounts of activated caspases between cells and supernatant could occur, the fluorescence was measured sequentially at 3 different time points (1, 2, 6 hours) after APO-ONE buffer/substrate addition to obtain reliable results.

As shown in Fig. 22, caspase 3/7 were strongly activated after treatment with all tested drugs. Remarkably, the caspase 3/7 activity was detected in the cells as well as in the supernatant indicating that after 24 hours treatment the apoptotic process was advanced. Taking a closer look one can see that OLO II and CP at a final concentration of 1 µM displays the same ability to induce apoptosis in human HL-60 leukaemia cells. Only VP-16, which seems to be especially effective in inducing apoptosis in HL-60, shows higher apoptotic potential. To induce apoptosis with the conventional Olomoucine much higher drug concentrations are required. In this sense, the effectiveness of 150 µM OLO is comparable with that exerted by 5 µM OLO II (**Fig. 22**).

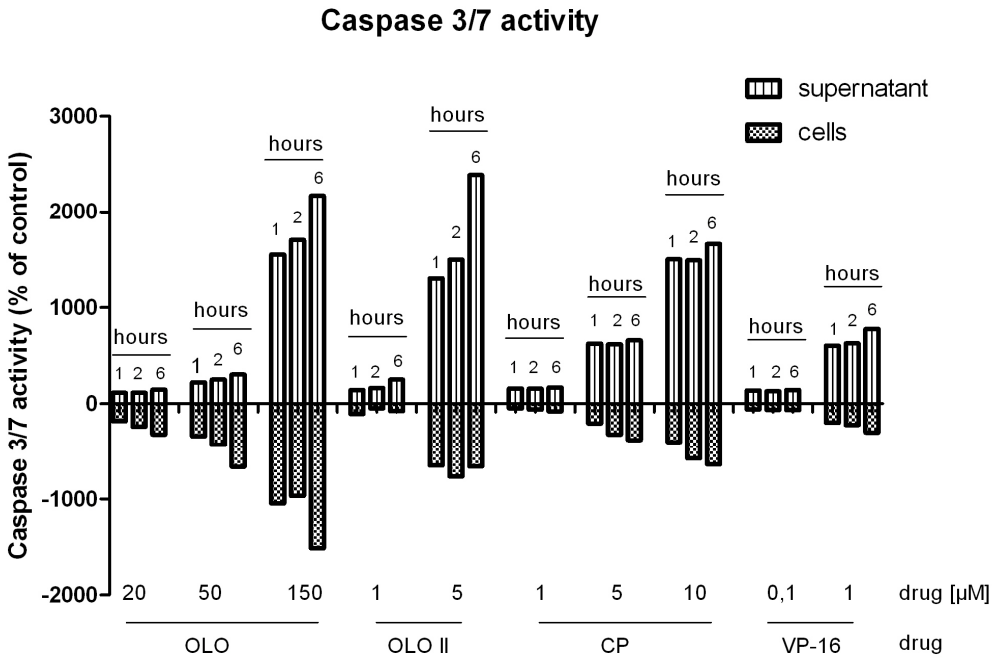

Figure 22. Increase of caspase 3/7 activity after treatment of HL-60 cells for 24 hour with CDK inhibitors. The upper half of the diagram represents caspase 3/7 activity measured in the supernatant; the lower part shows caspase 3/7 activity determined in the cells.

OLO and OLO II induce release of cellular lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from the action of chemical compounds. Cyto-TOX-ONE assay is a fluorometric method for estimating the number of non-viable cells by measuring the release of lactate dehydrogenase into the culture medium. Released LDH is measured using a coupled enzymatic assay that results in the conversion of resazurin into the

fluorescent resorufin. Quantification is achieved by dividing the amount of released LDH by the number of viable cells ascertained by a cell viability assay e.g. the previously used 'CellTiter-Glo Luminescent Cell Viability assay'. Finally, values obtained for distinct samples are normalized against that determined in untreated controls (% of control cells). As a positive control a sample obtained after lysis of control cells was used.

After treatment of HL-60 cells with OLO, OLO II, CP and VP-16 for 24 hours the LDH release was determined and again a strong induction of apoptosis by OLO and OLO II was detected (**Fig. 23**).

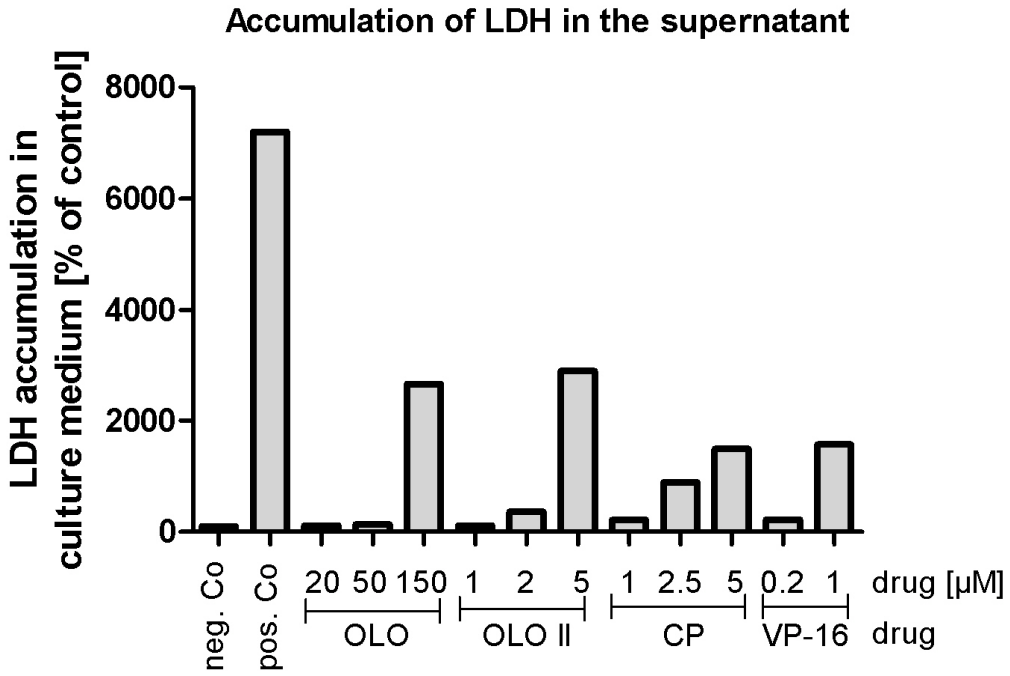

Figure 23. Accumulation of LDH in culture medium after treatment of HL-60 cells with distinct drugs for 24 hours

OLO and OLO II lead to loss of the potential of the mitochondrial membrane

Another method to detect apoptosis is the determination of the potential of the mitochondrial membrane by JC-1 staining. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. JC-1 is a fluorescent dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenimidazolylcarbocyanine iodide) that signals the loss of the mitochondrial membrane potential. The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye to enter the mitochondrial matrix where it accumulates and forms aggregates. However, in apoptotic cells JC-1 remains in the cytoplasm in its monomeric form. After excitation with UV light, the aggregated and monomeric form of JC-1 show different absorption/emission maxima that can be measured by flow cytometry in two

separate channels and distinguished by bivariate analysis. We performed JC-1 staining using HL-60 cells treated for 24 hours with OLO, OLO II, CP and VP-16.

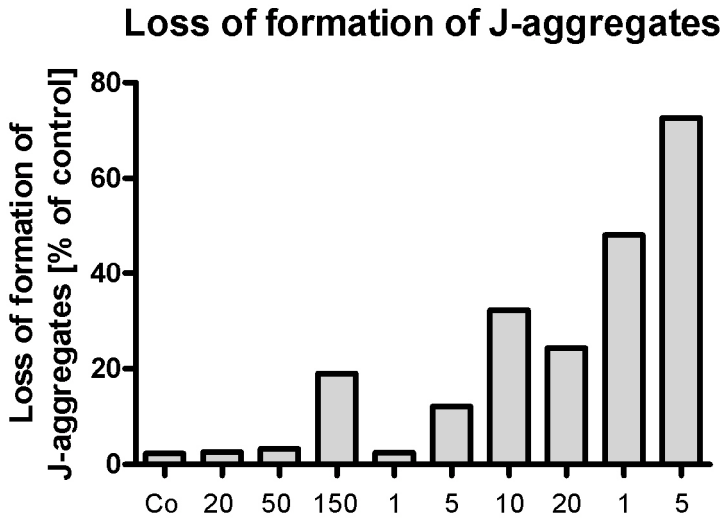

Figure 24. Loss of J-aggregate formation in HL-60 cells upon treatment with distinct drugs for 24 hours

Again we could confirm that the number of apoptotic cells resulting from treatment with 150 µM OLO equals the amount resulting from treatment with 5 µM OLO II or 10 µM CP. Etoposide (VP-16) still remains the most potent 'HL-60 killer' (**Fig. 24**).

OLO or OLO II treated cells accumulate 7-aminoactinomycin D (7-AAD)

Cellular accumulation of 7-AAD is a sensitive test for monitoring changes of the integrity of plasma membrane. Depending on its status, the 7-AAD dye is able to enter dead/late apoptotic cells, whereas it is excluded from live cells. This allows the discrimination between dead and live cells and their quantification by flow cytometry. Again, a marked uptake of 7-AAD was observed in HL-60 leukaemia cells after treatment with tested drugs for 24 hours (**Fig. 25**) indicating that the cells were in late stages of apoptosis.

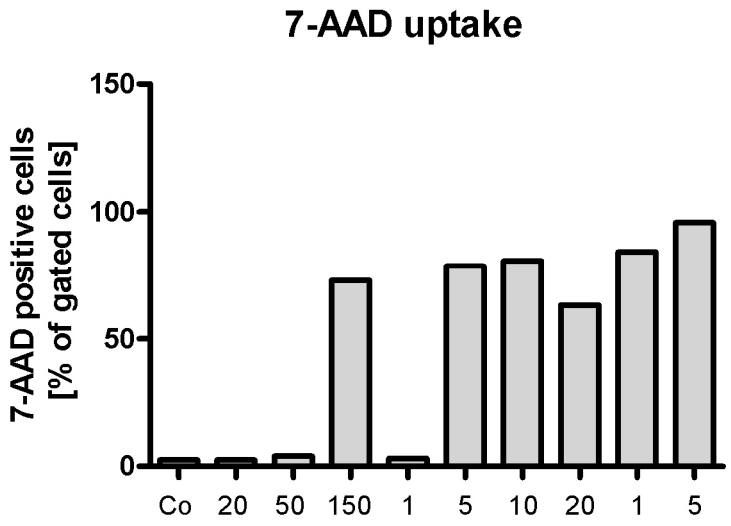

Figure 25. 7-AAD positive cells after drug treatment for 24 hours

Taken together all our results strongly indicate that the tested CDK inhibitors exert a strong pro-apoptotic potential on the human HL-60 leukaemia cells line. It is obvious, that Olomoucine II is more effective than Olomiucine and comparable with Cisplatin, a commonly used therapeutic agent. As already mentioned Cisplatin displays various serious side effects in the clinical use but is still widely use due to the lack of better, equally potent alternatives. Olomoucine II on the other hand has so far proven to be non-genotoxic, only slightly cytotoxic and is hence a very promising - only since very recently commercially available - pharmacological CDK inhibitor for the use in human acute myeloid leukaemia.

5.2 Effects of OLO, OLO II and ROSC on human cervical carcinoma cells ($HeLaS_3$)

The CKI Roscovitine (ROSC) is a potent inhibitor of cell cycle progression in asynchronously growing HeLa cells and is further able to induce apoptosis

As we had already observed that Olomoucine and Olomoucine II are able to induce a weak reversible cell cycle arrest and promote a strong apoptotic signal in human leukaemia cells, we decided to examine their effectiveness on human HeLa cervical cancer cells. Both human HeLa cervical cancer cells and human HL-60 leukaemia cells are fast dividing, exponentially growing and aggressive cells. However, HeLa cells are HPV-18 positive and therefore they escape from the proper cell cycle regulation due to the expression of HPV-encoded

oncoproteins. Considering this fact, it was important to prove whether CDK inhibitors would be able to inhibit proliferation of cells lacking the G_1/S checkpoint.

Previous results in our lab had already lead to the assumption that Olomoucine was indeed able to affect HeLa in much the same way as it affected HL-60 cells (data not shown). To further elucidate the effects of the inhibition of CDKs in HeLa cells, we raised two questions. First, we asked whether the tested pharmacological CDK inhibitors would be able to inhibit cell cycle progression and also induce apoptosis in synchronized cells, and if yes, in which cell cycle phase. As depicted in Fig. 26 Roscovitine induces a G_2 arrest at both used concentrations (20 and 40 µM) in asynchronously growing HeLa cells. It became evident that higher ROSC concentrations were necessary to initiate apoptosis. The number of hypoploid cells increased after exposure of cells to 40 µM ROSC for 6 hours and reached a maximum after further 12h.

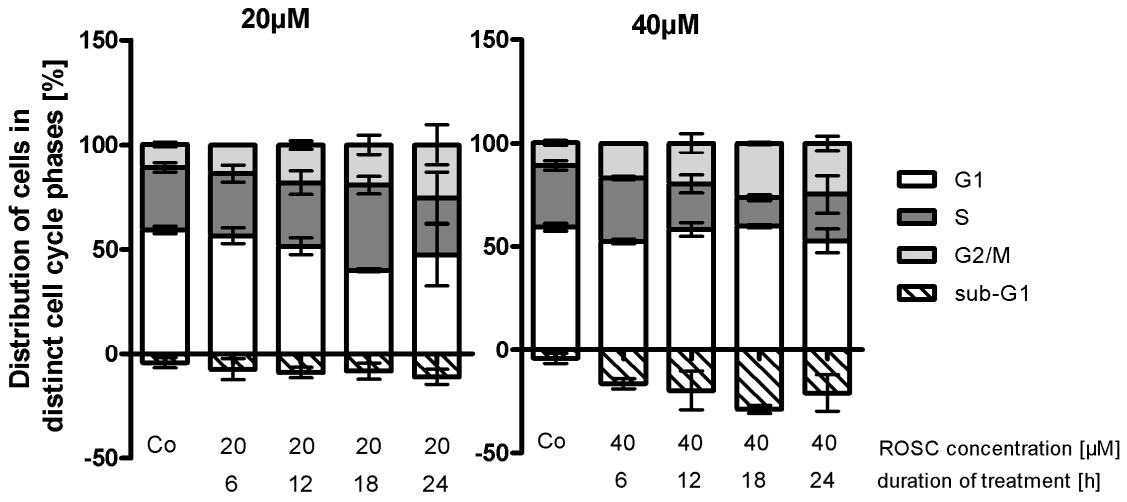

Figure 26. Effect of ROSC on the distribution of cells in distinct cell cycle phases after treatment of asynchronously growing HeLa cells

In parallel assays the frequency of apoptotic HeLa cells was determined using CytoDEATH M30 monoclonal antibodyies. The anti-serum directed against caspase-3-cleaved form of cytokeratin 18 stains selectively cells undergoing apoptosis. Interestingly, the highest number of CytoDEATH-positive HeLa cells was detected after treatment of HeLa cells for 12 hours (**Fig. 27**).

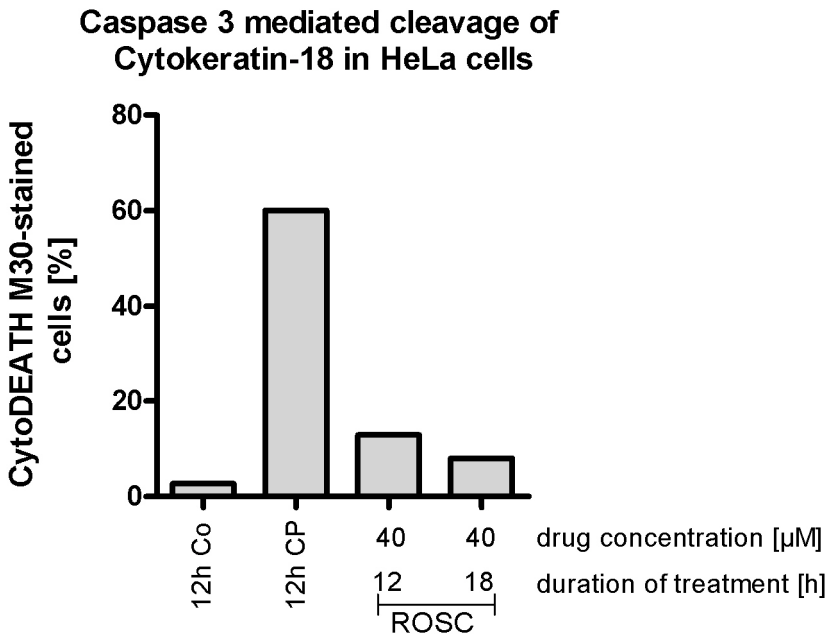

Figure 27. Quantification of caspase-3 cleaved cytokeratin 18 in HeLa cells after treatment with Cisplatin (CP) and Roscovitine (ROSC) using CytoDEATH M30 antibodies staining of cells. The cells were exposed to the drugs for 12 or 18 hours. The CytoDEATH M30 stained cells were quantified by flow cytometry.

The above results indicate that the dose-dependent outcomes of the treatment of HeLa and HL-60 cells with CDK inhibitors are similar.

Serum deprivation does not lead to cell cycle arrest in HeLa cells

One method to synchronize cells in G_1 phase is serum starvation. In the absence of mitogenic stimuli normal and some cancer cells become arrested in G_1 because they cannot progress through G_1 phase and cannot pass the G_1/S checkpoint. As HeLa cells do not have a functional G_1/S border due to E7-mediated inactivation of the retinoblastoma protein, one would expect low, if any, effect, on the cell cycle progression of HeLa after serum deprivation.

Our results confirmed the suspicion. Serum withdrawal didn't affect the distribution of HeLa cells in the cell cycle phases (**Fig.28**), thereby evidencing their independence from mitogens. Moreover, unlike in some cell lines, serum deprivation in HeLa cells didn't induce apoptosis.

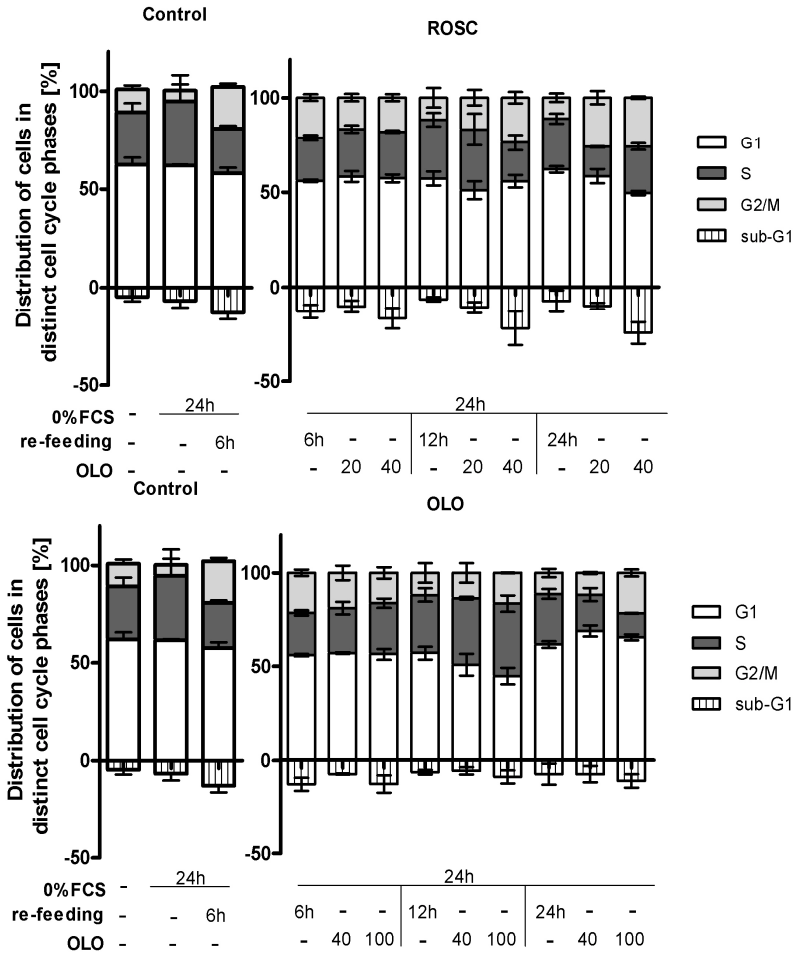

Figure 28. Cell cycle distribution after serum deprivation and OLO/ROSC treatment

Interestingly, release of serum-starved HeLa cells to FCS- and CDK -inhibitors supplemented medium didn't reveal any spectacular changes. Dosage-dependent accumulation of G_2-arrested and apoptotic cells resembled those observed after Olomoucine or Roscovitine treatment of asynchronously growing HeLa cells (**Fig. 28**).

OLO induces a G_2 arrest in HeLa cells released from Nocodazole-mediated mitotic block

In the next step HeLa cells were synchronized in G_2/M phase using Nocodazole, a microtubule disrupting drug. Nocodazole is a commonly used synthetic inhibitor of the microtubule polymerisation that reversible blocks the cell cycle in G_2/M phase (**Fig.29a and 30a**). After exposure of HeLa cells to Nocodazole for 18h, cells were accumulated in G_2/M phase (87 to 99 %) as evidenced by flow cytometric measurement of DNA concentration in single cells. Considering the fact that using this method it is not possible to distinguish between cells blocked in G_2- and M-phase, additional test were performed. Cells were stained with Hoechst to visualize chromatin and then inspected under fluorescence microscopy.

Using this method it was clearly visible that nocodazole-treated HeLa specimen were highly enriched in cells blocked in the mitosis. Then the spindle drug was removed and cells were released in the medium or directly to medium with Olomoucine or Roscovitine for 6, 12 or 24 hours.

The Nocodazole induced cell cycle block is reversible. After wash-out, cells released from mitotic block to medium progressed through the cell cycle and after 6 hours the population of cells in early G_1 phase reached about 80 %. 24 hours after the release from mitotic block the distribution of cells in distinct cell cycle phases was comparable with that of asynchronously growing cells (**Fig. 29a and 29b**). These results showed that shortly after wash-out of Nocodazole the population of HeLa cells is partially synchronized in the early G_1 phase of the cell cycle.

In the next experiments, the early G_1 phase-synchronized HeLa cells were exposed to OLO or to ROSC.

Interestingly, after 12 hours OLO (40 or 100 µM) transiently arrested HeLa cells in G_1 phase. After 24 hours an accumulation of G_2 or S-phase at 40 µM and 100 µM OLO, respectively, was observed (**Fig. 29b and 29c**). Moreover, after release of HeLa cells from nocodazole-induced mitotic block a population of apoptotic cells appeared. Treatment with OLO at both concentrations elevated the rate of apoptosis Fig. 29b and 29c.

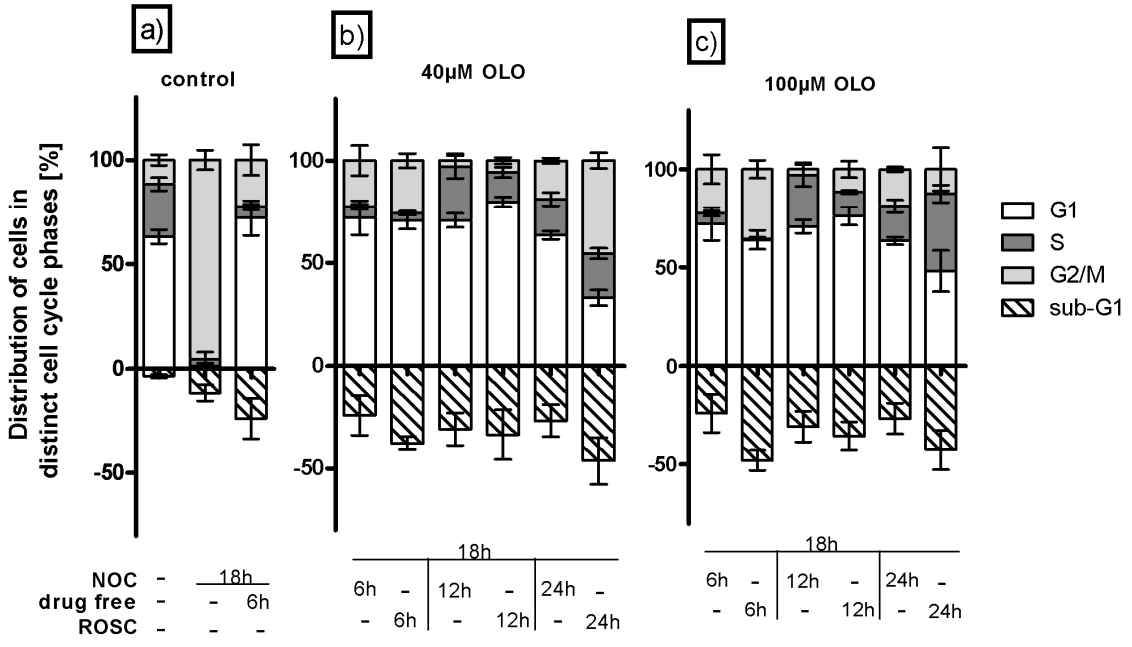

Figure 29. Cell cycle phase distribution after cell cycle block in G_2 phase using Nocodazole and subsequent treatment with OLO

Roscovitine strongly inhibits cell cycle progression in HeLa cells released from Nocodazole-mediated mitotic block

Release of HeLa cells from mitotic block directly into medium containing ROSC markedly affected the cell cycle progression. The observed changes were dose- and time-dependent (**Fig. 30b**). After treatment for 6 hours, ROSC partially prevented the progression of cells and an increase of G_2 cell population was observed. The G_2 cell increase was more pronounced at higher ROSC dosage and was accompanied by dramatic reduction of the S-phase cells (**Fig. 30b**). The exhaustion of S-phase cells was also observed during the next 12 hours thereby indicating that inhibition of CDKs in HeLa cells released from mitotic block strongly impairs the cell cycle progression.

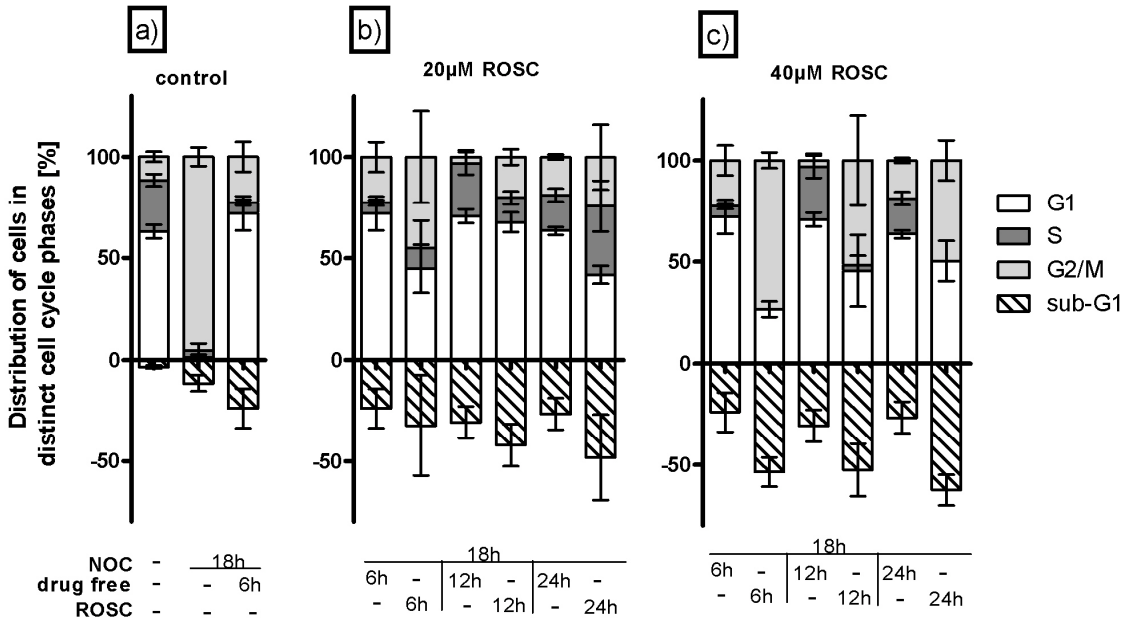

Figure 30. Effect of ROSC on the distribution of HeLa cells in distinct cell cycle phases after release of G_2 synchronized cells from Nocodazole-mediated block

Taken together, the effects exerted by CDK inhibitors on asynchronously growing cells, on cells released from serum starvation or from mitotic block strongly differ. The inhibition of cellular CDKs by two pharmacological inhibitors most pronounced interfered with the cell cycle progression. It remains to elucidate which CDK was functionally impaired after ROSC treatment.

5.3 Effects of OLO, OLO II and ROSC on immortalized and transformed rat fibroblasts

Temperature-sensitive p53 mutant induces G_1 arrest in rat cells maintained at permissive temperature

Human HeLa cells represent a good experimental model for cancer cells lacking the G1/S checkpoint due to E7-mediated Rb inactivation. Further, their p53 protein is not functional as a result of the E6-mediated ubiquitylation and degradation. Inactivation of both tumour suppressor proteins confer HeLa cells an enhanced and unlimited mitotic potential.

In the next studies another experimental model was used that allows comparing immortalized and highly transformed cells possessing the same genetic background. The immortalized and highly transformed rat cell clones were generated from primary rat fibroblast after constitutive expression of ts $p53^{135val}$ mutant alone or in combination with mutated c-Ha-Ras, respectively. Two immortalized cell lines designated 402/534 and 602/534 and two highly transformed cell clones termed 189/111 and 173/1022 were investigated. As a control, rat embryonic cells (REC) used for establishing the cell clones were used. Constitutively activated c-Ha-Ras is a characteristic of many tumours leading to growth factor independent cell proliferation.

To ensure that the established rat cell clones change p53 phenotype as predicted, cells were cultivated at a basal temperature (37°C) and then shifted to 32°C or 39°C. These cell lines switch in a temperature-dependent mode between phenotypes: wild-type p53 at 32°C, at 37°C predominantly mutated p53 and at 39°C exclusively mutant p53.

To determine their cell cycle distribution all four REC clones were cultivated at 32°C, 37°C and 39°C and DNA concentration in single cells was measured by flow cytometry. FACS analysis revealed that the cell cycle distribution of all the cell lines is temperature-dependent (**Fig. 31**). The p53-mediated cell cycle arrest became evident at 32°C as compared to primary rat fibroblasts 110/112 that express endogenous wild-type p53 at physiological level. A marked reduction in S phase and a prominent G_1 cell cycle arrest was observed in all four rat cell lines at 32°C. At 37°C the G_1 population is still frequent, but slightly reduced, especially in transformed cells. At 39°C an increase in S phase is associated with the reduction of the G_1 population– especially in 189/111 and 173/1022 cells.

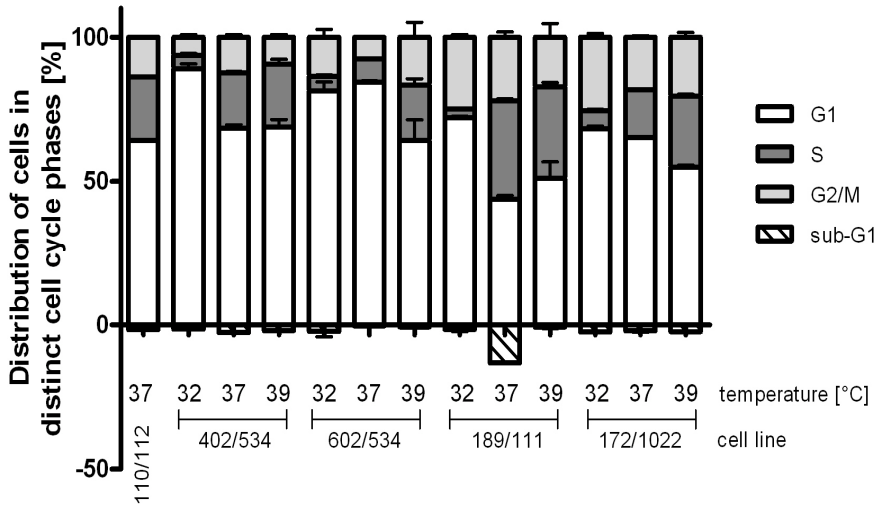

Figure 31. Cell cycle distribution at various temperatures

Temperature-dependent intracellular localization of ts p53^{135Val} mutant

To examine whether the temperature-dependent changes of the cell cycles are coupled to ts p53^{135Val} localization, we performed p53 staining using antibodies coupled to a flourochrome (see methods).

As expected, at 32°C p53 protein was localized in the nucleus were it can exert its functions as a transcription factor. However, at 39°C p53 was found in the cytoplasm (**Fig. 32**). These results confirm previously published data (Wesierska-Gadek et al. 1996) and indicate that p53 can exert its normal tumour suppressor function solely at the permissive temperature (32°C) but is unable to do so at elevated temperature (37°C and 39°C) .

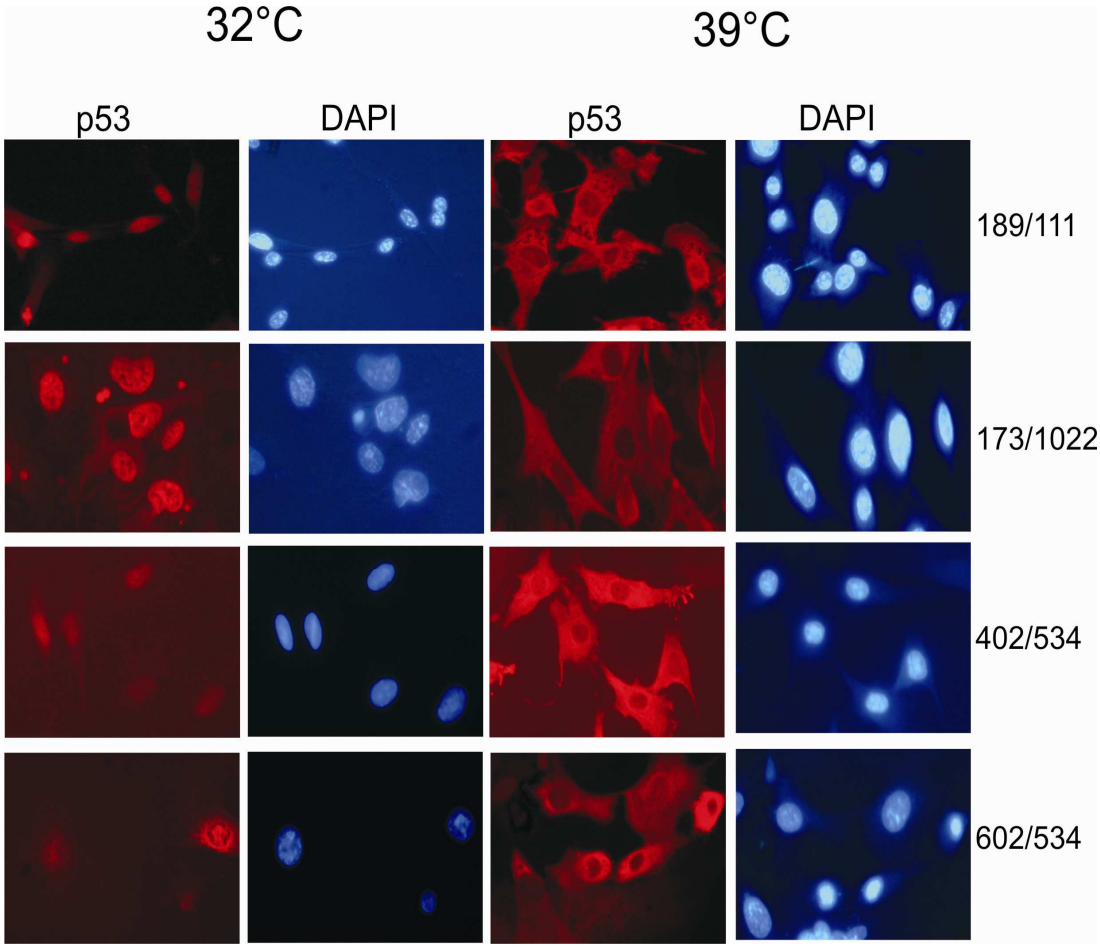

Figure 32. Immunostaining of RECs (189/111, 173/1022, 402/534, 602/534) maintained at 32°C or 39°C.
Chromatin was visualized by DAPI staining

At 37°C approximately 70 % of p53 protein is localized in the cytoplasm. In the primary rat cells 110/112 p53 protein expressed in low levels is exclusively localized in the nucleus at 37°C (data not shown).

Kinetics of $p53^{135Val}$-dependent G_1 arrest after shift to 32°C

As already mentioned $p53^{135Val}$ adopts wild-type conformation at the permissive temperature (32°C) and should hence be able to exert its role as 'the guardian of the genome'. As shown in Fig. 33, elevated $p53^{135Val}$ expression was observed in cells collected after cultivation at 32°C.

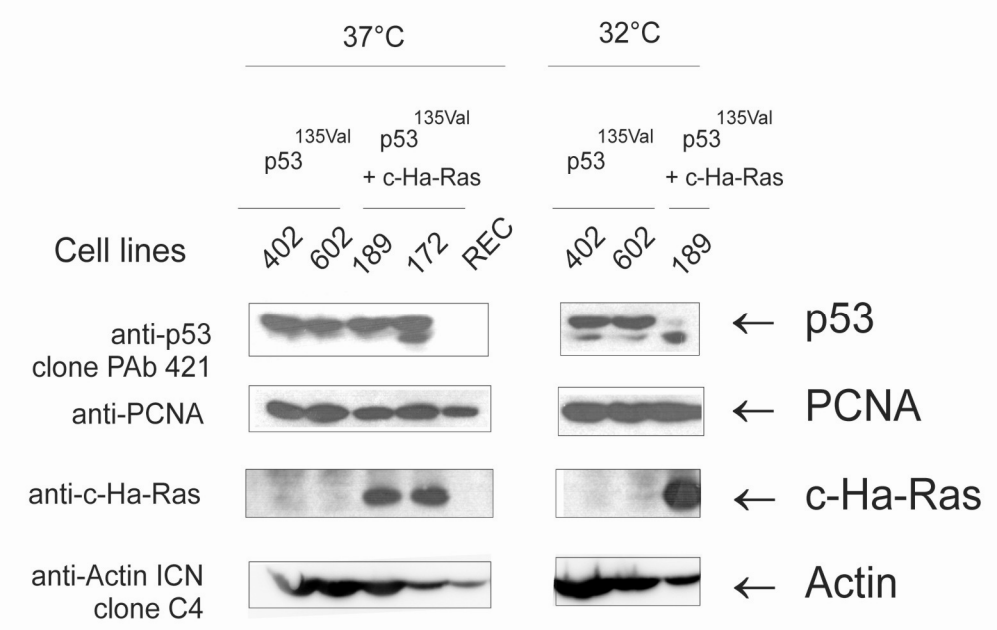

Figure 33. Immunoblotts of 189/111, 173/1022, 402/534 and 602/534 cells at 37°C or 32°C

As depicted in Fig. 34, the c-Ha-Ras transformed young (189/111) and old (173/1022) RECs arrest quite quickly after shift to 32°C. Already after 6 hours a slight G_1 arrest occurs that further increases after longer maintenance at the permissive temperature. The increase of the G1 cell population is accompanied by a marked reduction of S-phase in both cell lines. Interestingly, in the cell line 173/1022 the G_2 phase was markedly diminished after 24 hours (**Fig. 34**).

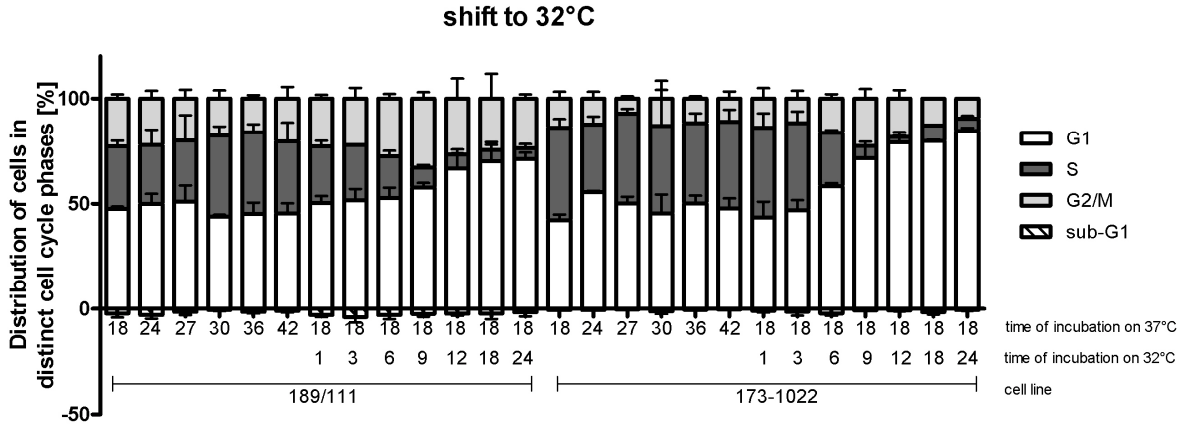

Figure 34. Cell cycle distribution of 189/111 and 173/1022 cells after temperature shift to 32°C

In immortalized cells 402/534 (young) and 602/534 (old) that proliferate much slower, the onset of p53-mediated cell cycle arrest was observed after 9 hours at 32°C. Moreover it

became obvious that 402/534 cells are more efficiently arrested in G_1 phase than 602/534 cells (**Fig. 35**).

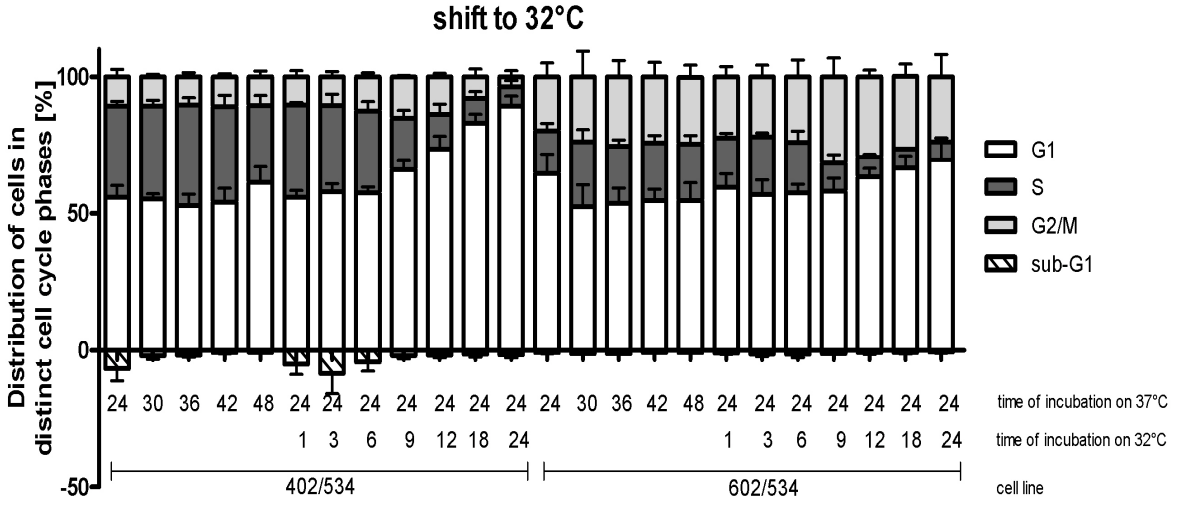

Figure 35. Distribution of 402/534 and 602/534 cells in distinct cell cycle phases of after temperature shift to 32°C

Summarizing the above data one may conclude that unlike the size of the S-phase in transformed cells cultivated at basal temperature outrun that in immortalized cells. Furthermore, there is a difference in the kinetics of cell cycle arrest between young and old cell clones generated from embryonic rat cells isolated from embryos at different time points of gestation (13.5 day versus 15.5 day). The observed difference is probably attributable to the differentially advanced development of the embryos.

In both transformed cell lines c-Ha-Ras is expressed at comparable levels.

p53-mediated cell cycle arrest is reversible

Further experiments confirmed that the p53-mediated cell cycle arrest initiated at the permissive temperature was reversible. Cells maintained for 24h at 32°C were shifted back to the basal temperature of 37°C. All tested cell lines re-enter the cell cycle (**Fig. 36**). Generally, transformed cells re-entered the active cell cycle more rapidly that immortalized cells.

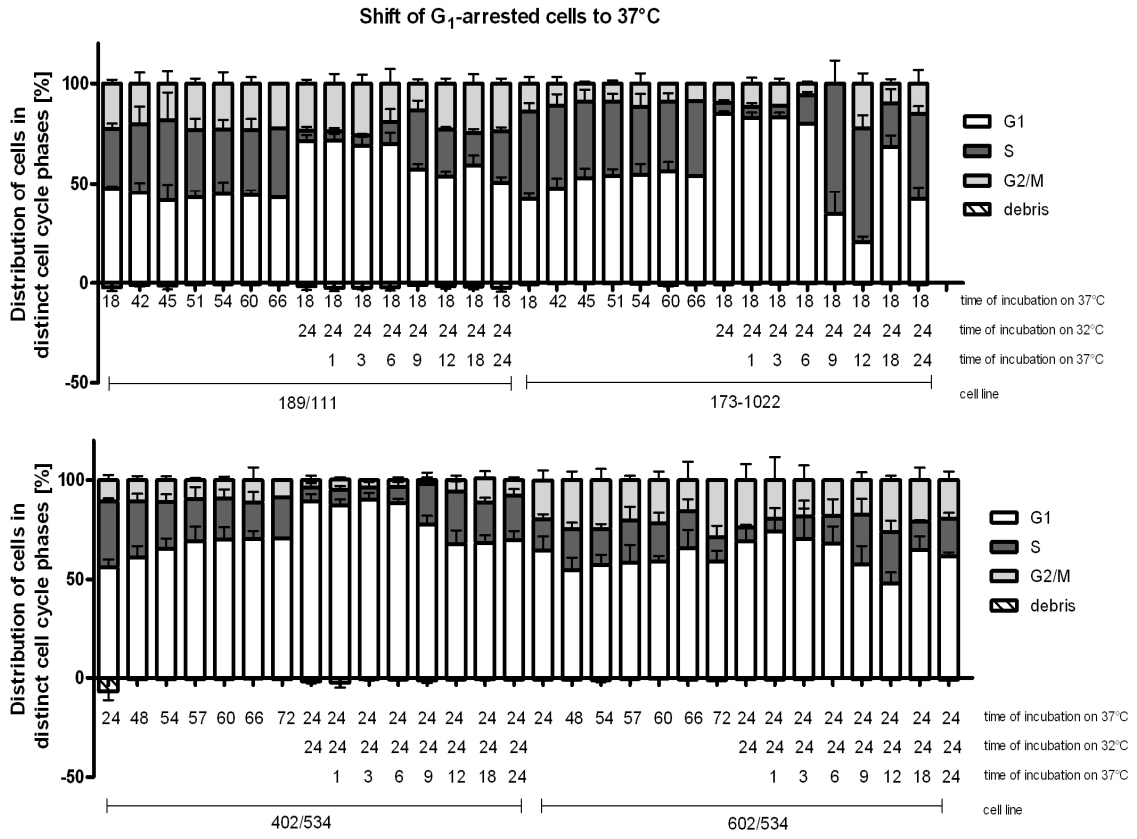

Figure 36. Distribution of rat cells in distinct cell cycle phases after back shift of G_1 arrested cells to 37°C

Again, different kinetics in the cell cycle recovery and progression between cell clones generated from young (189/111, 402/534) and old RECs (173/1022, 602/534) became evident (**Fig. 36**). These results strongly indicate that RECs isolated from embryos at 13.5 and 15.5 gestation day strongly differ in their development program and probably in the microenvironment.

A prolonged increase in S-phase in transformed cells maintained at 39°C

As already mentioned ts $p53^{135Val}$ protein fully adopts mutant conformation at 39°C. Furthermore, at 39°C ts $p53^{135Val}$ protein is exclusively localized in the cytoplasm and is hence inactive as a transcription factor (**Fig. 32**). Interestingly, this shift of the p53 conformational status has a marked impact on the cell cycle progression and cell proliferation primarily in transformed but at lower extent in immortalized cells (**Fig. 37**).

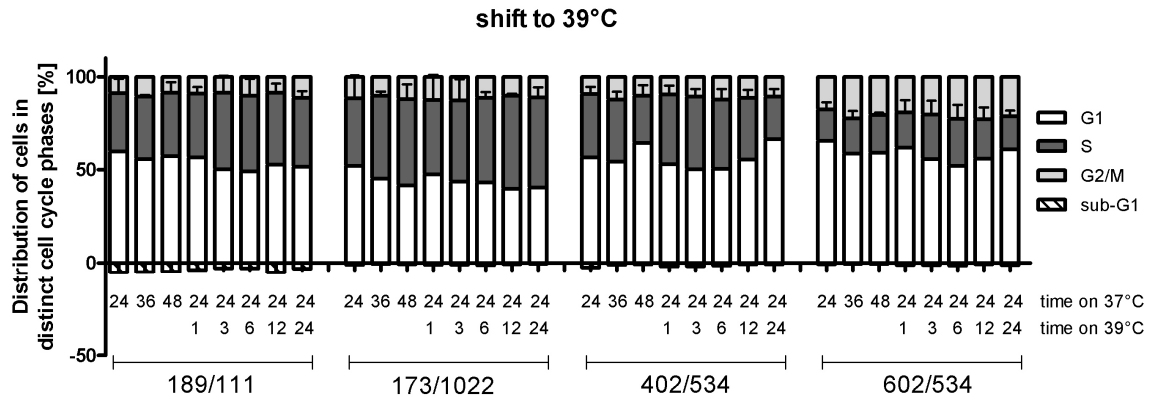

Figure 37. Cell cycle distribution after shift to 39°C

The strongest increase in S-phase was detected in transformed cell lines (189/111 and 173/1022 cells). In both immortalized cell lines (402/534 and 602/534) the accumulation of S-phase cells was transient and after 24 hours the frequency of the S-phase population was diminished. These results are consistent with the kinetics of cell proliferation and corroborate the assumption that overexpression of mutated c-Ha-Ras cooperates with mutant p53 protein and confer enhanced mitotic potential to the cells.

Olomoucine weakly affects cell proliferation of all four tested REC cell lines

After establishing how overexpressed ts $p53^{135Val}$ and c-Ha-Ras affect the cell cycle progression in the RECs, we examined how the tested cells would respond to treatment with the CDK inhibitors Olomoucine, Olomoucine II and Roscovitine. All three drugs have proven to be useful in cancer therapy in my previous experiments.

First, the effects of these drugs on the cell proliferation of the RECs were examined. Cells were continuously exposed to increasing concentration of the drugs for 24 hours or 48 hours at 37°C. After expiration of this time period 'CellTiter Viablity Assay' was performed as described previously (see methods). As shown in Fig. 38a, Olomoucine only weakly affected cell proliferation of the REC cell lines after 24 hours treatment. The cell lines 173/1022, 189/111 and even the non-transfected cell line 402/534 only showed a 20 % decrease in cell number. The immortalized cell line 602/534 showed a stronger decrease in cell number (by 10 %). After 48 hours of treatment the inhibitory effect was slightly stronger, except for 173/1022 cells. The cell line 173/1022 remained almost unaffected.

In summary, Olomoucine was unable - up to a final concentration of 100 µM - to inhibit the proliferation of the examined rat cell lines.

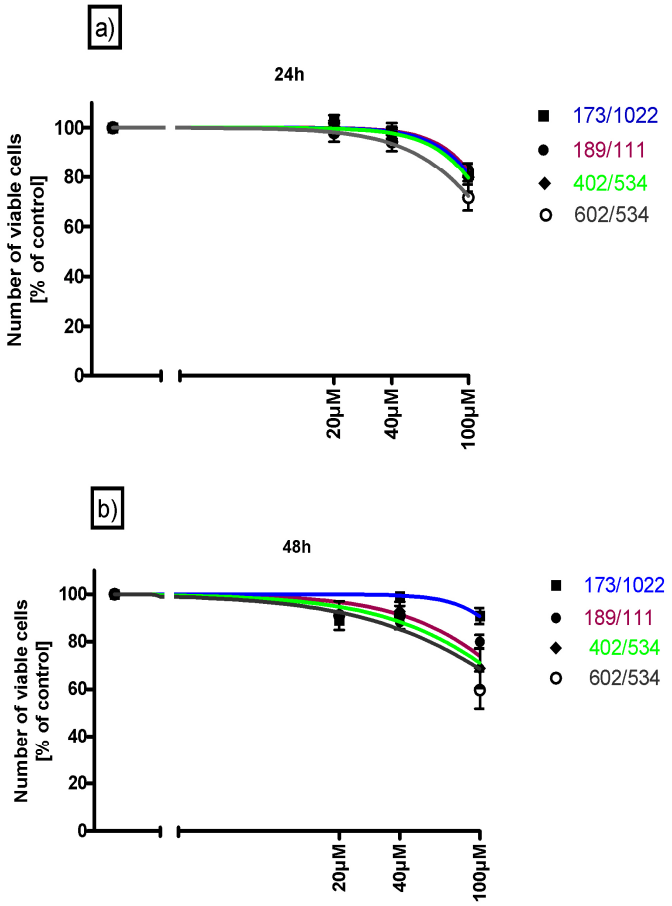

Figure 38. Effect of OLO on the proliferation of rat cells after continuous treatment for 24 (a) or 48 (b) hours

Unlike OLO, OLO II had a stronger effect on the tested RECs cell lines. Again, immortalized cells were more susceptible to the treatment for 24 hours than transformed cells (**Fig. 39a**). After 48 hours the number of viable cells was reduced by approximately 10 %, except for the transformed cell line 173/1022 (**Fig. 39b**). Interestingly, the immortalized 402/534 cells overexpressing solely tsp53^{135Val} were most sensitive to the action of OLO II, the IC_{50} was 9.7 µM OLO II. The IC_{50} for 602/534 was not reached at the used concentrations.

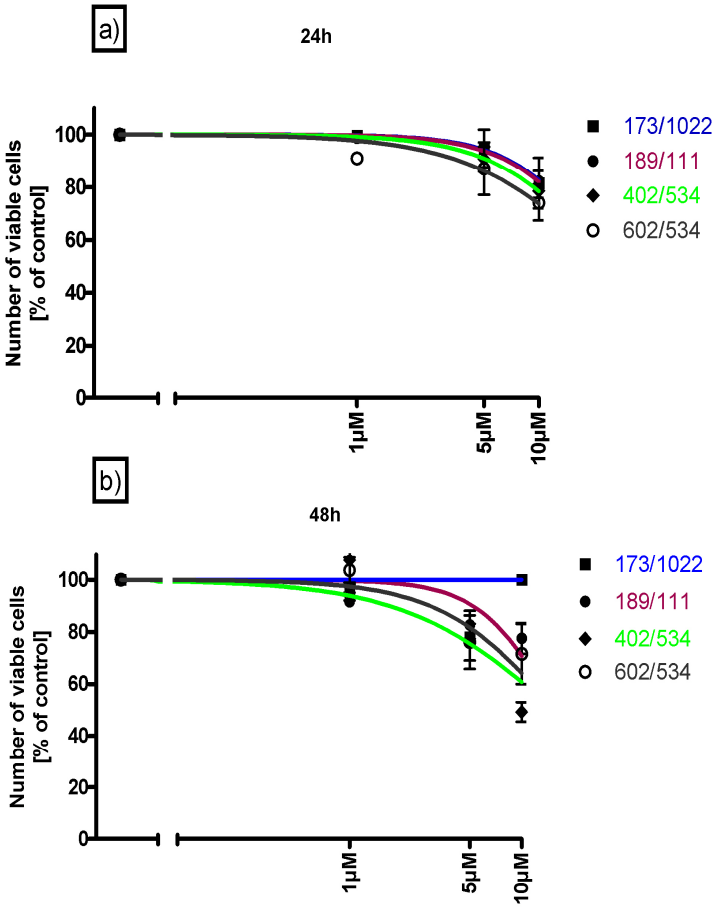

Figure 39. Effect of the continuous treatment for 24 (a) or 48 (b) hours of RECs with OLO II on REC cell lines

Finally, rat cell lines were treated with Roscovitine. The susceptibility of all tested cell lines to Roscovitine was comparable. The drug reduced the number of living cells by 30 % within 24 hours and after further 24 hours by additional 10% (**Fig.40a**). Roscovitine exerted the strongest effect on immortalized 402/534 cells (**Fig. 40b**).

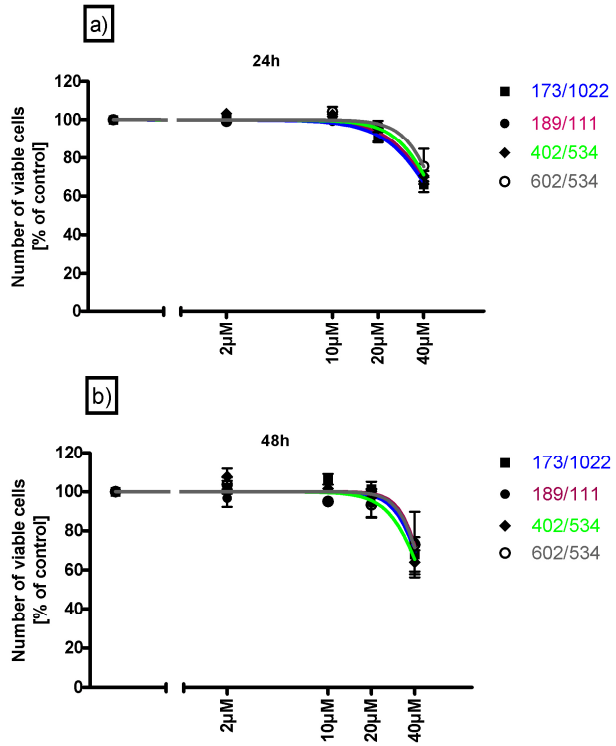

Figure 40. Effect of ROSC on REC cell lines after continuous treatment for 24 (a) or 48 (b) hours

To assess statistical significance of the results, One-way-ANOVA analysis and the 'Dunnette post test' were performed (**Fig. 41**).

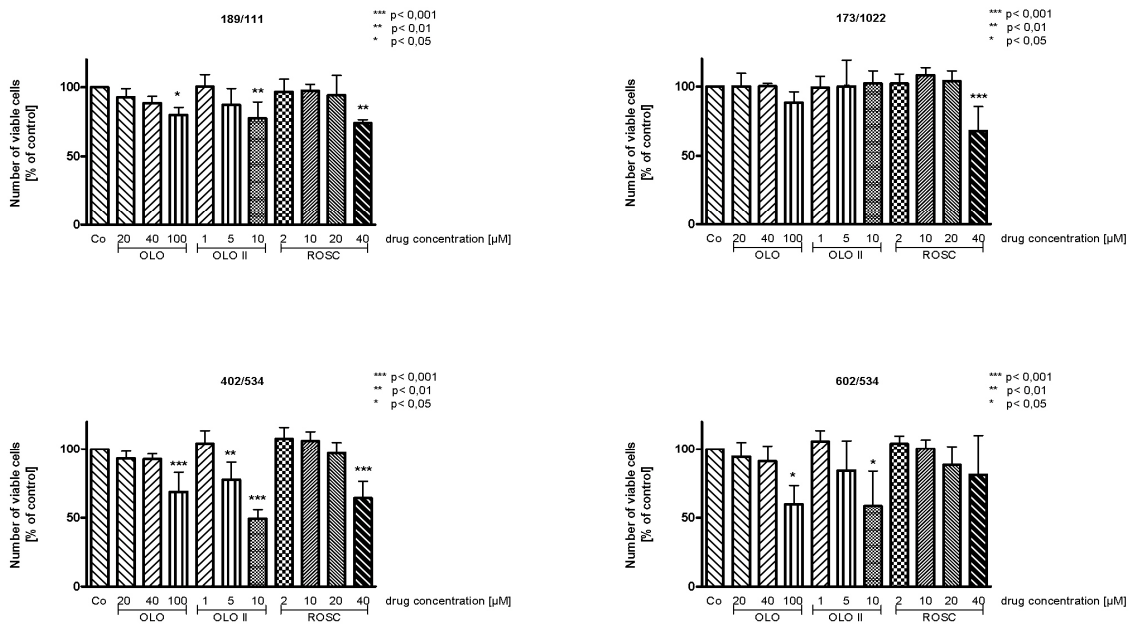

Figure 41. Evaluation of the statistical significance of the effect of CDK inhibitors (OLO, OLO II and ROSC) on tested rat cell lines upon treatment for 48 hours

Used statistic test: One-way-ANOVA, Dunnett post test

Taken together, the results clearly show that ROSC and OLO II have stronger growth-inhibitory potential than OLO. Furthermore, it is obvious that immortalized cells are much more sensitive to the action of ROSC and OLO II than transformed cells. Interestingly, immortalized cells were stronger affected by OLO II than by ROSC, whereas in the case of transformed cells the inverted trend was observed. These results indicate that the overexpression of c-Ha-Ras renders cancer cells less sensitive or even resistant to the action of pharmacological CDK inhibitors.

The relative low efficacy of the examined CDK inhibitors on the proliferation of transformed rat cells brought us to determine their effects on the cell cycle progression.

The conventional Olomoucine only weakly affects the cell cycle progression of the transformed rat fibroblast cell lines 189/111 and 173/1022

Both transformed rat cell lines were cultivated in medium containing Olomoucine for 12, 24 or 48 hours. After treatment for 12 hours a transient increase in G_2 cells was observed in both cell lines (**Fig. 42**). A slightly higher accumulation of the ratio of G_2 phase cells occurred in 189/111 rat cells. Remarkably, after longer exposure, 189/111 cells responded to OLO with an increase of G_1 phase, whereas 173/1022 cells with a slight accumulation of S-phase cells (**Fig. 42**). Inspection of the DNA histograms revealed that in OLO-treated cells hypoploid cells appeared. The higher ratio of sub-G_1 cells was detected after treatment with OLO at a final concentration of 100 µM. In conclusion, Olomoucine has a very weak effect on the cell cycle progression of 189/111 and 173/1022 cells.

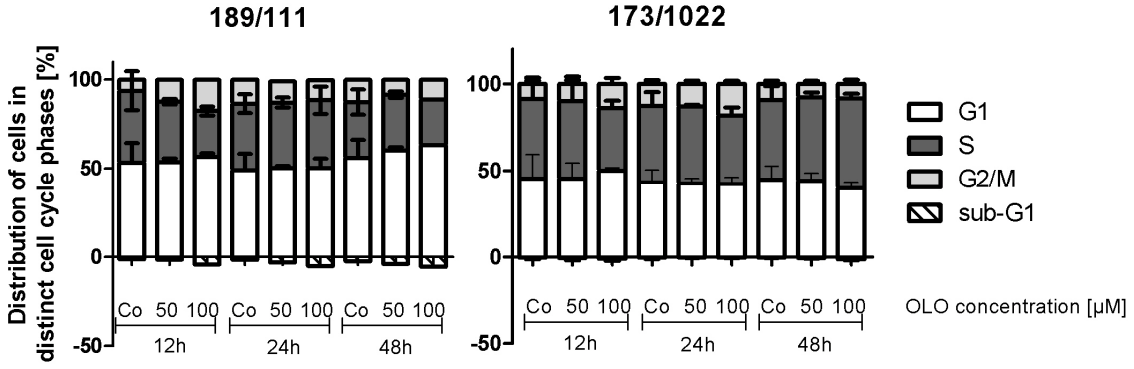

Figure 42. Effects of OLO on the cell cycle distribution of 189/111 and 173/1022 cells

The cell cycle progression of 402/534 cells (young RECs) is a little stronger affected by Olomoucine than that of 602/534 cells (old RECs)

Unlike transformed rat cells, immortalized rat cells were a little more affected by the action of OLO. In 402/534 cells the changes of the distribution of cells in distinct cell cycle phases after Olomoucine treatment for 12 and 24 hours almost resembled that observed in 189/111 cells (**Fig. 43**). In 402/534 cells the ratio of S-phase cells was generally lower than in 189/111 cells. No effect was detected after treatment with Olomoucine for 48 hours (**Fig. 43**).

Like 173/1022 cells, 602/534 cells were only marginally affected by Olomoucine but a slight increase of the G_2 ratio – especially at high (100 µM) drug concentration – was still detectable (**Fig. 43**).

It is very interesting that the trend of the changes of the cell cycle distribution observed after Olomoucine treatment of 189/111 (young RECs, p53, c-Ha-Ras) and 402/534 (young RECs, p53) cells is similar. Similarity was also found between 173/1022 (old RECs, p53, c-Ha-Ras) and 602/534 (old RECs, p53) cells. Thus, it seems that the intrinsic features of the primary cells isolated from rat embryos at gestation day 13.5 or 15.5 are essential determinants partially overriding effects of oncogene *RAS*.

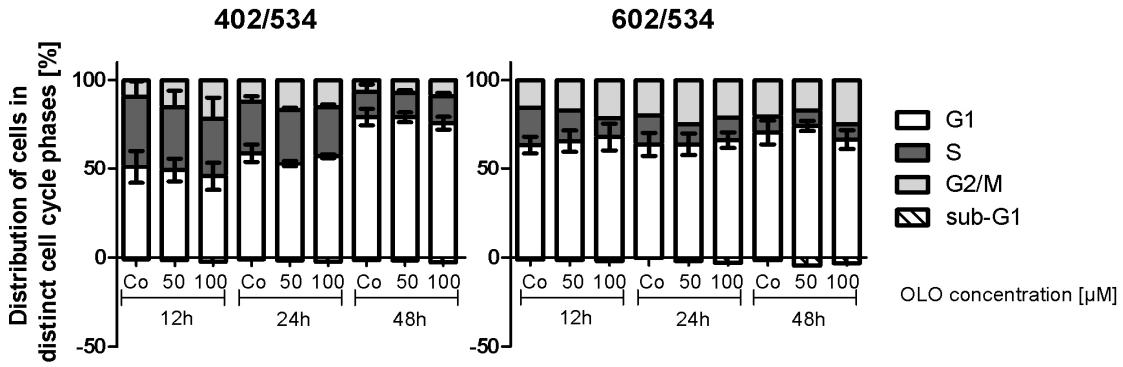

Figure 43. Effects of OLO on the cell cycle distribution of 402/534 and 602/534 cells

Olomoucine II differentially affects cell cycle progression of immortalized and transformed rat cells

Unlike OLO, OLO II induced changes of the cell cycle in transformed rat cells. Remarkably, 189/111 cells were stronger affected than 173/1022 cells. OLO II at higher concentrations (10 µM) increased the ratio of G_2 phase cells within the period of treatment (12 hours to 48 hours) (**Fig. 44**). Moreover, after 48 hours OLO II resulted in the reduction of the ratio of S-phase cells. This was accompanied by the appearance of hypoploid cells. Similar changes but at lower extent were induced in 173/1022 rat cells (**Fig. 44**).

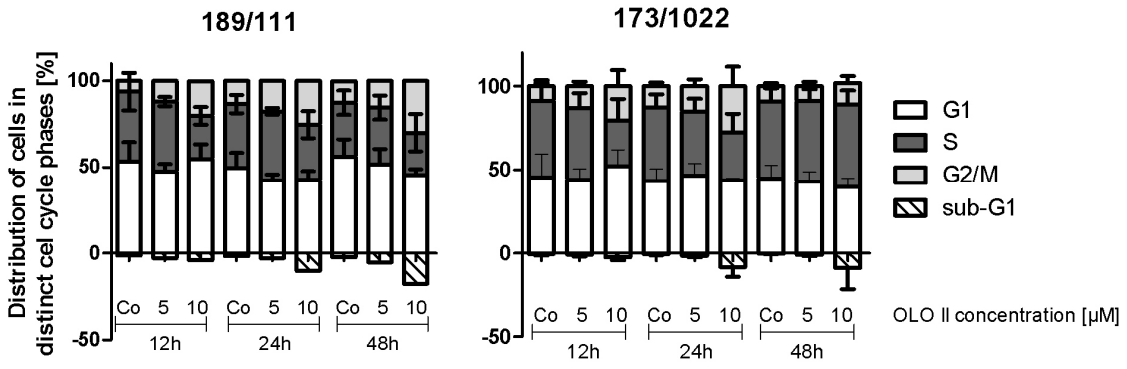

Figure 44. Effects of OLO II on the cell cycle distribution of 189/111 and 173/1022 cells

In contrast**,** OLO II strongly affected the progression of the cell cycle of immortalized cells (**Fig. 45**). In 402/534 cells an accumulation of G_2-arrested cells was accompanied by a reduction of S-phase cells (**Fig. 45**). After treatment for 48 hours a population of hypoploid cells appeared. It can further trigger apoptosis even though not as strong as in 189/111 cells. 602/534 cell cycle progression is only mildly affected by Olomoucine and apoptosis can barely be detected in those cells (**Fig. 45**). In 602/534 cells the outcomes altogether were detectable but much weaker (**Fig. 45**).

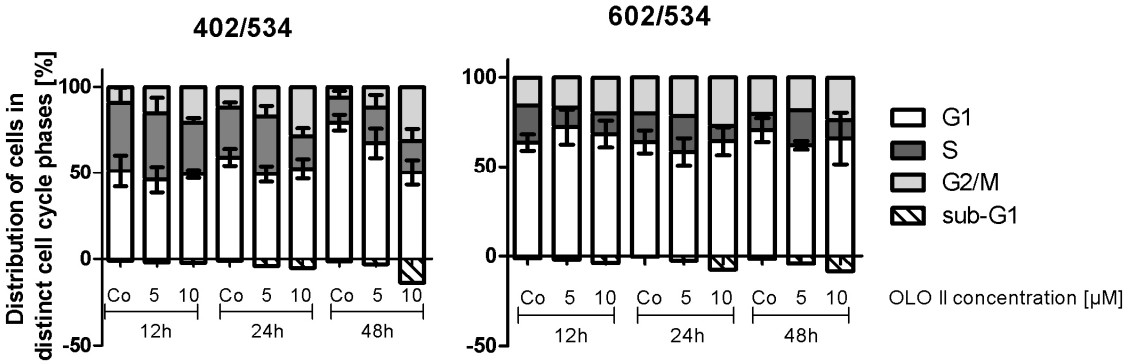

Figure 45. Effects of OLO II on the cell cycle distribution of 402/534 and 602/534 cells

Again, the young RECs (402/534, 189/111) display similar patterns of cell cycle changes after drug treatment. On the other hand, the response of the immortalized old RECs (602/534) and transformed old RECs (173/1022) differed.

Roscovitine has a stronger effect on cell cycle progression of transformed cells than OLO II

Finally, the action of ROSC on the cell cycle of transformed rat cells was determined. The pattern of the cell cycle changes were similar to those observed after OLO II treatment. These results were surprising because OLO II usually displays much stronger cell cycle inhibitory effects in human cancer (**Fig. 46**).

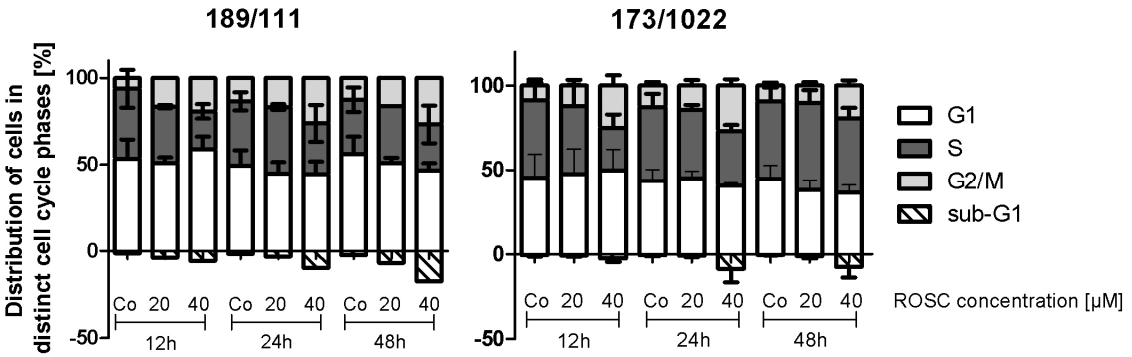

Figure 46. Effects of ROSC on the cell cycle distribution of 189/111 and 173/1022 cells

Roscovitine has a weak effect on cell cycle progression of immortalized rat cells

ROSC slightly increased the ratio of S-phase and G_2 cells in immortalized rat cells 402/534 within the first 24 hours. At higher ROSC concentration S-phase was diminished, as compared with untreated control. After treatment for 48 hours sub-G_1 cells appeared (**Fig. 47**).

In contrast, 602/534 cells were barley affected by ROSC (**Fig. 47**).

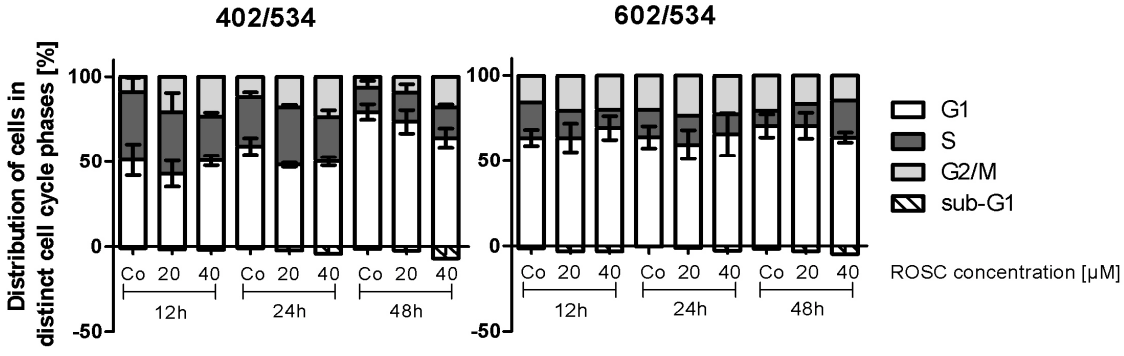

Figure 47 Effects of ROSC on the cell cycle distribution of 402/534 and 602/534 cells

The appearance of hypoploid cells especially in cultures of transformed rat cells (189/111 and 173/1022 cells) after exposure to pharmacological inhibitors of CDKs indicated that the drugs

initiate apoptosis. To ensure that apoptosis but not necrosis was induced, the activity of the effector caspases was determined using APO-ONE assay.

Activation of effector caspases 3 and 7 in transformed rat cells

Flow cytometric analyses of DNA content revealed an appearance of hypoploid cells upon 24 hour/or longer treatment of transformed rat cells. Therefore, it was reasonable to examine the activity of the effector caspases in both transformed rat cell lines but not in immortalized cells. APO-ONE assay in 189/111 and 173/1022 cells was performed after treatment for 24 hours (**Fig. 48**) and for 48 hours (**Fig. 49**). The assay confirmed the assumption that ROSC and OLO II induce apoptosis. Thus, the population of sub-G_1 cells seems to represent cells undergoing apoptosis. As shown in Fig. 48, ROSC and OLO II activated caspases 3/7 four-fold and eight-fold, respectively, as compare with the untreated control cells. Furthermore, it became evident that the transformed rat cell line 189/111 is more sensitive to the drugs than 173/1022. In the latter, the enhancement of the activity of effector caspases was very negligible. The results also show that Roscovitine and Olomoucine II are more potent inducers of apoptosis than Olomoucine.

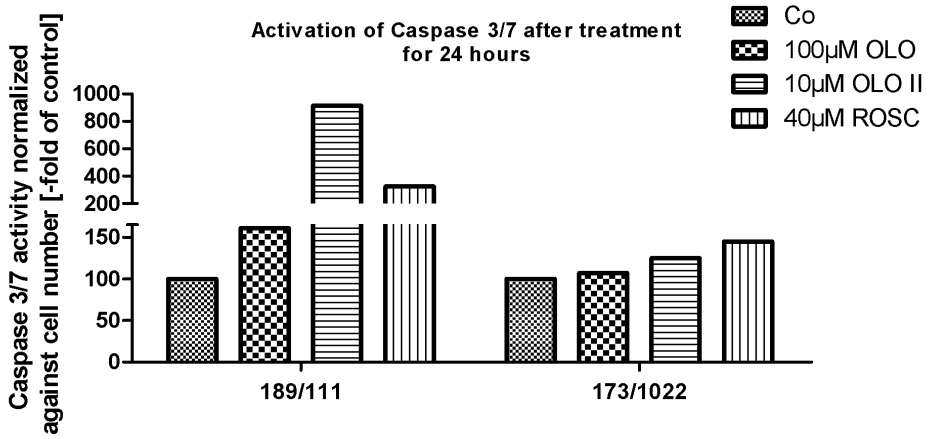

Figure 48. APO-ONE assay of 189/111 and 173/1022 cells after 24 hours of drug treatment

The different sensitivity of rat cells to CDK inhibitors was also detected after continuous treatment for 48 hours (**Fig. 49**). In this series of experiments the activity of the effector caspases was determined in all four REC cell lines. As depicted in Figure 49, the CDK inhibitors induced apoptotic proteases after treatment for 48 hours primarily in transformed cells. In the two immortalized cell lines the activity of caspases 3/7 increased only negligibly. This corresponds well with the low ratio of hypoploid cells. However, in transformed cells the activation of caspases-3/7 after exposure to CDK inhibitors became obvious. All three drugs induced the activity of effector caspases. Much stronger activation of apoptotic proteases was

observed in 189/111 cells than in 173/1022 cells. The stronger activation of effector caspases in 189/111 cells confirms the results of flow cytometric determination of DNA content and reflects their higher sensitivity to CDK inhibitors very well. In 402/534 and 602/534 cells, cells not expressing mutant c-Ha-Ras unlike 189/111, apotosis is barely induced indicating that the reduction in cell number detected with 'CellTiter Viability Assay' is probably mainly due to cell cycle arrest and not due to apoptosis.

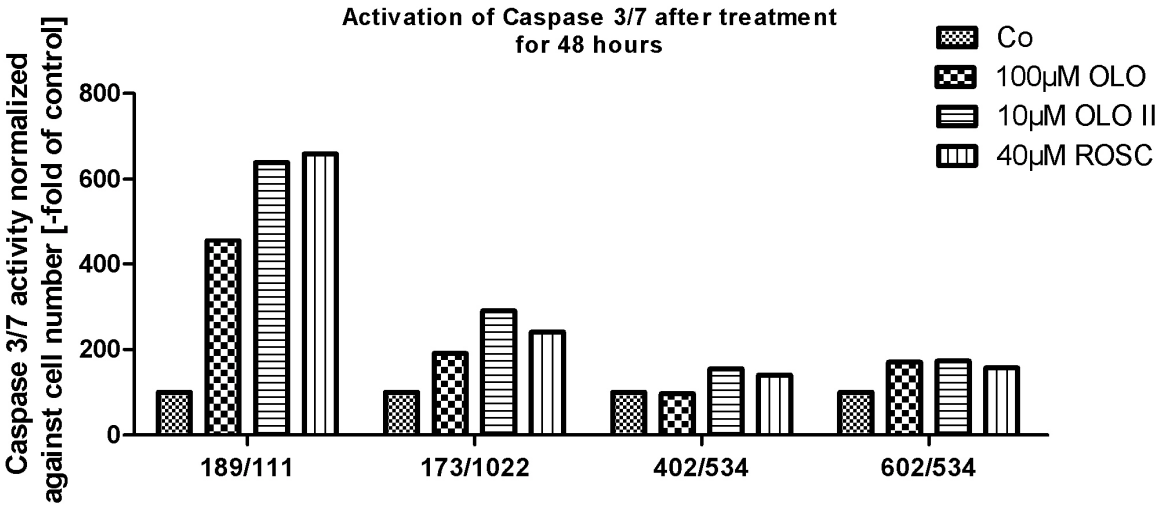

Figure 49. APO-ONE assay of 189/111, 173/1022, 402/534 and 602/534 fibroblasts after 48 hours of drug treatment

Wt p53 sensitizes transformed rat cells to CDK inhibitors-mediated apoptosis

All the above presented date were ascertained after treatment of cells maintained at a basal temperature (37°C), it means under conditions at which cells overexpressing ts $p53^{135Val}$ display mainly mutant p53 (~70 %).

Therefore, to further assess the impact of the p53 status on the outcome of drug treatment, transformed and immortalized rat cells maintained were exposed to the drugs at the permissive temperature of 32°C. As already shown in the previous experiments at 32°C p53 protein adopted a wild-type conformation resulting in a transient cell cycle arrest and a stop of cell proliferation.

Interestingly, wt p53 sensitized transformed cell lines to the tested CDK inhibitors. However, the outcomes of the inhibition of CDKs differed between the two transformed cell lines. The presence of high levels of wt p53 protein enhanced the apoptosis in only one (189/111) out of the two examined transformed cell lines. 189/111 cells were arrested in the G_1 phase of the cell cycle at the onset of treatment. The apoptosis rate was dose-dependent. The highest ratio of sub G_1 cells was detected after exposure of 189/111 cells to ROSC. Surprisingly, OLO at lower dosage induced apoptosis at high rates. Remarkably, the expression of wt p53 protein in

173/1022 didn't promote apoptosis but enhanced the inhibition of the cell cycle. All three CDK inhibitors markedly reduced the ratio of the S-phase cells and simultaneously increased the size of the G_1 cell population. Surprisingly, the strongest G1 arrest was induced by OLO at a final concentration of 100 µM. These results evidence that the intrinsic features of cells and their programme are able to override, at least partially the action of oncogenes and of tumour suppressor genes and determine the susceptibility of cells to therapy.

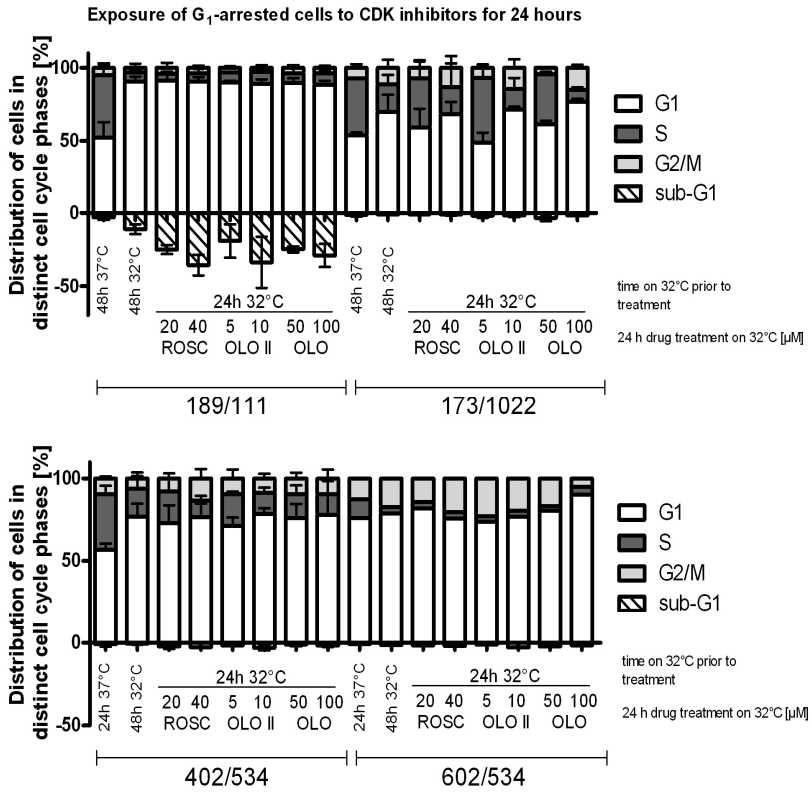

Figure 50. Cell cycle distribution of the REC cell lines 189/111, 173/1022, 402/534 and 602/423 after drug treatment on 32°C

In summary, it was demonstrated that the susceptibility of cancer cells to therapy may be modulated by the p53 tumour suppressor protein and by oncogenic c-Ha-Ras. Transformed cells expressing wt p53 protein generally display increased sensitivity to the action of CDK inhibitors, whereas expression of oncogenic Ras protein renders them resistant to the CDK inhibitors. Furthermore, it became evident that the sensitivity of immortalized cells to treatment with CDK inhibitors resembles that of normal healthy cells. These observations confirm the increased sensitivity of cancer cells to the therapy by pharmacological CDK inhibitors and substantiate the selectivity of their action. Furthermore, the results of flow cytometric analyses of DNA content pointed out some marked differences in the distribution

of cells in distinct cell cycle phases after drug treatment between cell clones generated from embryonic cells isolated at day 13.5 or 15.5 of gestation.

During our experiments marked differences in the 'behaviour' of 189/111 and 173/1022 cells were found. Considering the fact that these cells were isolated from embryos at different days of gestation, it appears likely that these differences might be attributable to the differences in signalling pathways responsible for embryonic development. As the hedgehog pathway is one of the major pathways involved in embryonic development and is known to strongly interact with Ras signalling, the effect of the inhibition of hedgehog signalling was examined using Cyclopamine, a specific Smothened inhibitor. The action of Cyclopamine was tested at three different concentrations (1, 5, 10 µM) in the two transformed rat cell lines.
Our preliminary results didn't reveal any dramatic impact on the cell number and cell cycle progression after treatment for 24 hours (data not shown). However, after continuous exposure for 48 hours or longer, the difference in the susceptibility of cell clones was observed. These preliminary data are encouraging and the experiments will be continued to rule out in more detail the involvement of hedgehog signalling.

5.4 Effect of OLO, OLO II and ROSC on normal human MRC-5 fibroblasts

Roscovitine and Olomoucine only weakly affect the cell number of healthy MRC-5 fibroblasts after continuous treatment for 24 hours

All the above presented data strongly evidenced therapeutic action of Olomoucine, Olomoucine II and Roscovitine in a variety of cancer cell lines. To attest the tested CDK inhibitors increased selectivity towards transformed cells, their action on normal healthy cells was of great importance. Human MRC-5 cells fulfil the criteria and are good experimental model. These cells divide moderately; grow much slower after approximately 40 population doublings and stop to divide after further 4-5 population. They exit from the active cell cycle and become senescent.
Using ´CellTiter Viability Assay´ we determined the effects of Olomoucine, Olomoucine II and Roscovitine on human MRC-5 lung fibroblasts. As depicted in Fig. 50, Olomoucine (**Fig. 51a**) and its successor Olomoucine II (**Fig. 51b**) do not or only weakly reduce the cell number of MRC-5 cells after 24 hours of treatment. Roscovitine does reduce cell number by 25 % if used at high concentrations (80 µM) (**Fig. 51c**).

Even after subsequent cultivation in a drug-free medium for 48 hours Olomoucine does not lead to a significant decrease in cell number. Cells treated with Olomoucine II or Roscovitine according to this schedule display markedly reduced cell numbers. In both cases the half maximum inhibitory concentration (IC_{50}) was reached. For OLO II the IC_{50} is 5.17 µM and for ROSC its 53.46 µM hence in both cases, especially for ROSC, the dose required to achieve the IC_{50} is probably higher than the dosage that will ever be used in clinic.

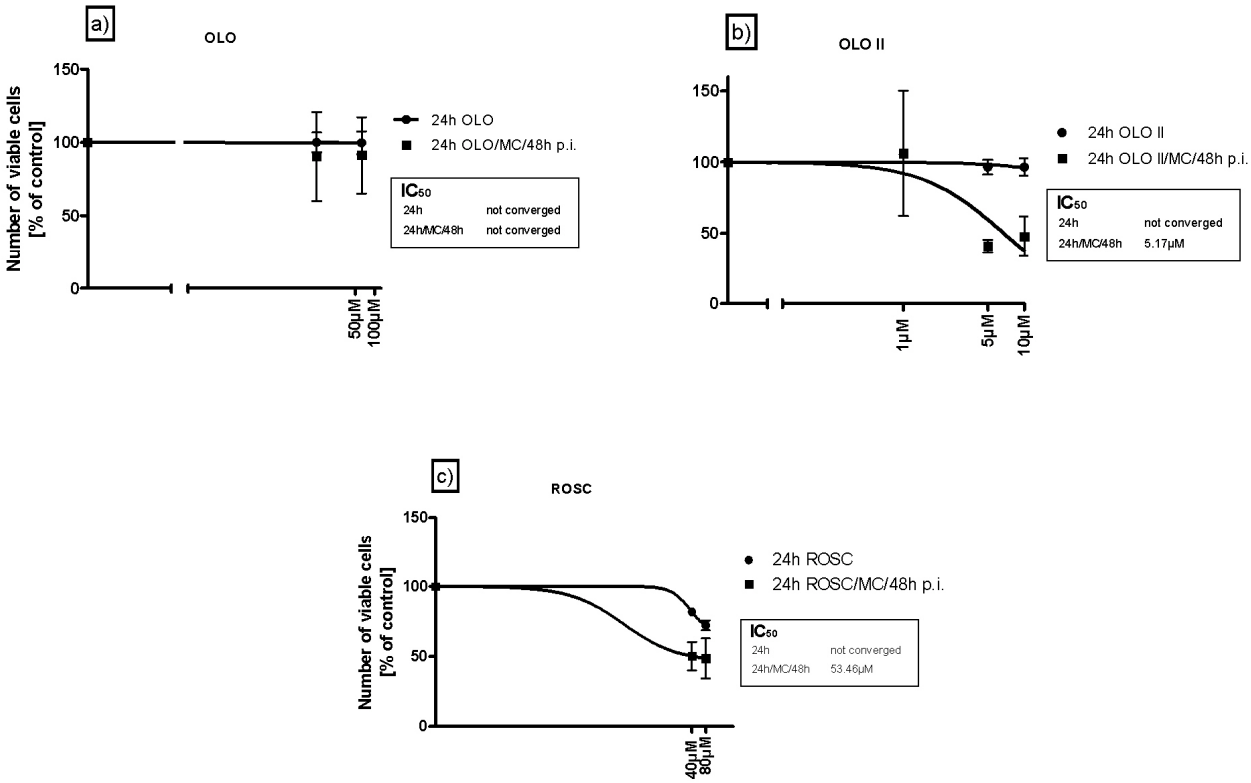

Figure 51. Effects of OLO, OLO II and ROSC on MRC-5 proliferation

Drug	IC_{50} [µM]
OLO	Not achieved
ROSC	53.46
OLO II	5.17

Table 3. Comparison of IC_{50} values for MRC5 cells after treatment for 24 hours with CDK inhibitors and post-incubation for 48 hours in a drug-free medium

Olomoucine, Olomoucine II and Roscovitine induce a slight G_2 arrest but not apoptosis in normal human MRC-5 cells

All of the used drugs induce a slight G_2 arrest in MRC-5 cells but fail to induce apoptosis (**Fig. 52**). The strongest G_2 arrest induced by Roscovitine correlates well with the results obtained by ´CellTiter Viability Assay´ (**Fig. 51**).

In summary, Olomoucine, Olomoucine II and Roscovitine inhibit the cell cycle progression of human MRC-5 fibroblasts within 24 hours but do not induce their death.

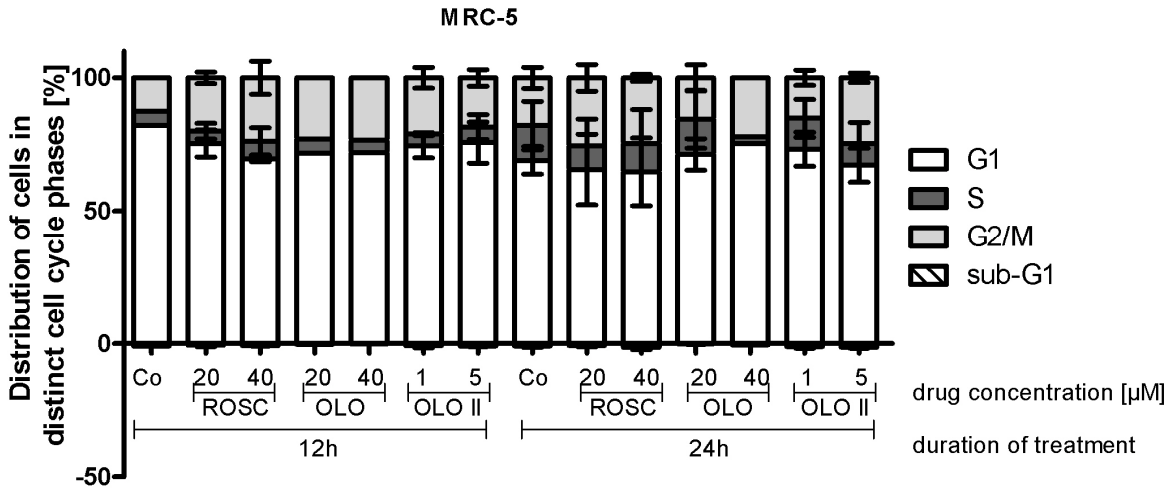

Figure 52. Effect of OLO, OLO II and ROSC on the distribution of MRC-5 cells in distinct cell cycle phases.

5. Discussion

Cancer cells develop form normal, healthy cells during a multistep process called malignant transformation or carcinogenesis. The inactivation of cellular inhibitors of CDKs and of tumor suppressor proteins as well as the up-regulation and constitutive activation of oncogenes and factors accelerating cell proliferation and promoting cell survival strongly interfere with proper cell cycle regulation. As a result of the escape from the control of the cell cycle, cells acquire new features and the advantage of unlimited growth capacity. The malignant transformation can be initiated by multiple environmental factors such as chemical carcinogens (e.g. arsenite or nitrosamines), biological carcinogens (e.g. viruses such as the human papilloma virus (HPV) or human hepatitis B virus (HBV), physical carcinogens (e.g. UV, ionizing radiation) and personal genetic factors. [136] Thus, genetic and epigenetic alterations can lead the way from growth limited cells to highly invasive cells. [137]
The cell cycle is a key cellular pathway affected by those alterations. Especially, the constitutive activation or deregulation of its major components - CDKs and cyclins – [138] can lead to malignant processes. [139]
In the light of the above mentioned facts, targeting the cell cycle became a very promising approach for anti-cancer therapy. For this purpose pharmacological inhibitors of CDKs were developed. There are at least five different classes of compounds that have been shown to inhibit CDKs and thus constrain tumour cell proliferation due to various mechanisms under *in vitro* and/or *in vivo* conditions. [140]
One class of CDK inhibitors namely substituted purine analogues show higher specificity and selectivity than other CDK inhibitors. Since they are not genotoxic and only slightly cytotoxic, they do not generate secondary tumours like other chemotherapeutics. [141]
The CDK inhibitors Olomoucine, Olomoucine II and Roscovitine were used for my experiments and all of them are substituted purine analogues competing with ATP for the ATP binding cleft of CDKs. [142] Purine analogues were shown to be effective as anti-leukemic agents [143, 144] as well as agents for treatment of solid tumours. [145
During my thesis the effects of the above mentioned purine analogues on cell lines with different p53 and c-Ha-Ras status was investigated.
p53 was named ‘the guardian of the genome’ due to its uttermost importance and its ability to control a wide range of functions in an unstressed cell. Moreover, wt p53 protein is able to initiate a distinct number of processes in response to cellular stress. It is involved in nucleotide excision and base excision repair via transcriptional regulation or direct interaction

with DNA repair factors. [146] p53 can further induce transient cell cycle arrest or completely stop proliferation (senescence). [147] If cell cycle arrest or senescence are no longer an option owing to severe, irreparable DNA injury etc., p53 induces apoptosis in damaged cells. p53 protein can promote apoptosis via the intrinsic (mitochondrial pathway) and extrinsic (death receptor mediated) pathway and the regulation of various pro- and anti-apoptotic proteins. [148]

During my diploma thesis we used cells with different p53 background in order to determined the importance of the p53 status of a cell prior to drug treatment for the outcome of drug treatment.

Human myeloid HL-60 leukaemia cells were used as a model for cells of p53 null genotype. Human cervical carcinoma cells (HeLa S3) as a cell line displaying wt p53 genotype but not functional p53 protein due to human papilloma virus protein E6 mediated degradation. However, it has been observed that p53 protein can be reactivated in the latter after treatment with CDK inhibitors [46]. The human MRC-5 fetal lung fibroblast cells display both wt p53 genotype and normal functional p53 protein

Apart from a complete loss of *p53* in the genome of HL-60 cells and decreased stability of p53 protein in HeLa S3 cells, both cell lines display also other kinds of abnormalities. For example, HL-60 cells exhibit an approximately 15-30 fold amplification of c-myc as compared to normal cells [113] and HeLa S3 cells lack functional Retinoblastoma protein (Rb) and consequently the checkpoint at the G_1/S border. [109]

Human myeloid HL-60 leukaemia cells seem to be more sensitive do the action of CDK inhibitors than HeLa cells. However, these cells are apoptosis prone and undergo much easier apoptotic cell death than other cancer cell lines. For these reasons it is difficult to evaluate whether the differential sensitivity of both examined cell lines to the pharmacological CDK inhibitors is attributable e to their differences in G_1/S checkpoint and p53 status.

Therefore, we used primary rat embryo cells overexpressing a temperature-sensitive murine p53 mutant (substitution from alanine to valine at position 135) alone or in combination with oncogenic Ras. Whereas cells overexpressing ts $p53^{135Val}$ were immortalized, those cell lines expressing concomitantly mutated c-Ha-Ras were highly transformed and after injection generated large tumours in rats within 2 weeks.

Using this temperature-sensitive $p53^{135Val}$ experimental model, we were able to asses the impact of p53 status on the sensitivity of cells to distinct drug treatments. Depending on the temperature of cell culture, cells display wt p53 protein at 32°C, but mutant p53 protein at 37°C and 39°C. Treatment of cells displaying wt or mutant p53 protein under the same genetic background gave us a powerful tool to evaluate the importance of p53 status for the

efficacy of the therapy. Finally, we studied the rat cell clones generated in primary rat embryonic cells isolated from embryos at different developmental stages (at 13.5 and 15.5. gestation day) in order to assess a relationship between sensitivity of cells to the therapy and the stage of differentiation. Using this experimental model it was possible to compare the susceptibility of transformed cells of less and more advanced differentiated origin. This allowed us to investigate whether strong oncogenes like e.g. oncogenic Ras are able to override the intrinsic cellular program.

The four rat fibroblast cell lines in short:

- cells overexpressing temperature-sensitive p53 isolated at day 13.5 gestation (402/534)
- cells overexpressing temperature-sensitive p53 isolated at day 15.5 gestation (602/534)
- cells overexpressing temperature-sensitive p53 + mutant c-Ha-Ras isolated at day 13.5 gestation (189/111)
- cells overexpressing temperature-sensitive p53 + mutant c-Ha-Ras isolated at day 15.5 gestation (173/1022/)

All thee above mentioned cell lines (HL-60, HeLa, MRC-5, 402/534, 602/534, 189/111, 173/1022) were treated with the pharmacological CDK inhibitors Olomoucine, Olomoucine II and Roscovitine. The CDK inhibitor Olomoucine specifically inhibits the cell cycle dependent kinases 1 and 2 [152], Olomoucine II and Roscovitine likewise inhibit CDK 1 and 2 [127, 128] but additionally inhibit CDK7 a kinase involved both in the cell cycle and transcriptional regulation. [63] CDK7-cyclin H in a complex form thee so called CDK activating kinase (CAK) that is required to activate other CDKs responsible cell cycle progression via phosphorylation in their T-loops. [46] Further CDK7 and cyclin H were shown to associate with the general transcription factor TFIIH which phosphorylates the carboxy-terminal domain (CTD) of RNA Polymerase II. [149] Therefore, inhibition of CDK7-cyclin H complexes has a dual consequence. It prevents its function as CAK and thereby the activating phosphorylation of all CDKs regulating the cell cycle and simultaneously leads to the inhibition of RNA Pol II and results in interruption of primary RNA transcript elongation and in consequence a global block of transcription. This transcriptional inhibition primarily affects proteins with fast turn-over rates and overexpressed proteins like inhibitors of apoptosis (IAPs) that are frequently overexpressed in cancer. Due to this transcriptional inhibition of inhibitors of apoptosis, the anti- and –pro-survival factors balance can be restored often leading to enhanced susceptibility of cancer cells to chemotherapeutics that induce apoptosis. Our results evidence that Olomoucine II and Roscovitine stronger affect the proliferation and the cell cycle of cancer cells than Olomoucine. Moreover, tested human cancer cells are more

susceptible to Olomoucine II than to Roscovitine. Rat transformed cells overexpressing mutated Ras are more resistant to the CDK inhibitors.

Like other cancer cells human leukaemia HL-60 cells display various abnormalities. One of them is a deletion of the p53 tumour suppressor gene. The loss of p53 alone is already a prerequisite for malignant growth but to make matter worse HL-60 further harbour multiple copies of the *myc* genes leading to 15-30 % amplification. Under normal physiologic conditions *myc* is required as a transcription factor for quiescent cells to re-enter the cell cycle, angiogenesis, stem cell renewal/differentiation etc. [150] If amplified *myc* often leads to uncontrolled growth and proliferation. [151] Those and other abnormalities lead to immortalization of HL-60 cells and contribute to the formation of this very aggressively growing tumour cell line.
Even though human leukaemia is a very aggressive malignancy, HL-60 due to p53 deletions, we were still able to induce G_2 cell cycle phase arrests and most importantly apoptosis in response to Olomoucine and its successor Olomoucine II. Apoptosis was induced in a concentration-dependent manner *via* activation of the effector Caspases 3/7 and was still detectable after complete removal of the drug from the culture medium and subsequently cultivation in a drug-free medium. Further, loss of membrane integrity and permeabilization of the mitochondrial membrane was detected in response to 24 hour treatment. This points out a long term effect that indicates major changes taking place within the cells. Intriguingly, Olomoucine II is able to induce apoptosis at around 10-fold lower dosage than Olomoucine. Whether this is a consequence of a strong inhibitory action on CDK7-cyclin H and thereby the transcription of various anti-apoptotic proteins e. g. survivin or is attributable to other completely different factors, has to be further elucidated.

HeLa cervical carcinoma cells are another 'model cell line' for aggressively growing cancers. They usually respond poorly to treatment with chemotherapeutics due to HPV-18 protein E6 mediated ubiquitination and subsequent degradation of p53 protein. Further in HeLa cells the Retinoblastoma (Rb) protein is inactive because HPV-18-encoded E7 leads to its sequestration thereby rendering it unable to bind members of the E2F transcription factor family which in turn allows constant activation of E2F transcriptional targets hence cell cycle progression. Taken together HeLa cells have lost p53 protein hence a major regulator of cell cycle arrest, DNA repair and apoptosis in response to cellular stress and their G_1/S checkpoint is abrogated due to Rb inactivation. Except for the fact that HL-60 cells arise form myeloid precursors and HeLa cells from epithelial ones, they both display similar abnormalities.

As expected, both Olomoucine and especially Roscovitine were able to induce cell cycle arrests in HeLa cells, just like in HL-60 cell. Interestingly the induced cell cycle arrest seems to be dependent on the cell cycle status prior to treatment and on drug concentration.
At lower doses Olomoucine and Roscovitine mainly induced a G_2 arrest in asynchronously growing cells. Cells synchronized in mitosis with spindle drug Nocodazole that were subsequently treated with Roscovitine or Olomoucine (especially at high concentrations) directly arrest in G_2 hence were unable to recover from the Nocodazole induced block. On the contrary cell that were synchronized with Nocodazole but released form the block for 4 hours prior to subsequent Roscovitine treatment arrest in G_1 instead of G_2 which is very intriguing as HeLa cells don't have a functional G_1/S checkpoint due to their lack of functional p53 protein. Further the same increase of cells in G_1 was detectable in asynchronously growing cells after 12 hours of Roscovitine treatment. Immunoblotting allowed us to clarify, that p53 was reactivated around this time and that the virally encoded proteins E6 and E7 were starting to be repressed allowing p53 reactivation in the first place. Further evidencing the reactivation of the G_1/S checkpoint is the decreased phosphorylation of CDK2 targets like Nucleophosmin (NPM). [152]
At higher concentrations of Roscovitine Caspase-3 mediated cleavage of Cytokeratin-18, an indicator for apoptosis, took place in response to Roscovitine treatment. The phosphorlyation status of two proteins involved in pro-apoptotic signalling namely Bad and Survivin was abrogated in response to high Roscovitine further indicating by which mechanisms Roscovitine is able to induce apoptosis. [152]

Rat fibroblast cell lines derived from the same genetic background but isolated at different days of gestation (13.5 or 15.5) transfected with different proteins namely temperature sensitive p53 or temperature sensitive p53 in combination with mutated c-Ha-Ras were used as a model for immortalized or highly transformed cells.
Performing histochemical staining we were able to show that p53 is located in the nucleus at the permissive temperature of 32°C whereas it is miss-localization to the cytoplasm hence separated form its target genes at 39°C.
If cultivated at 32°C for 24 hours all four rat fibroblast cell lines transiently arrested in G_1 (the arrest is revered if cell were subsequently transferred to 37°C) probably due to the abnormally high number of constantly expressed p53 protein in the nucleus. Under normal circumstances overexpression of p53 often leads to the induction of apoptosis due to activation of p53 down stream targets such as Puma, Survivine, Noxa etc. As already mentioned before, Roscovitine is able to induce apoptosis in HeLa cells due to abrogated Survivine phosphorylation etc.

Interestingly apoptosis could only be detected in 189/111 cells (isolated at day 13.5 gestation, expressing p53 and c-Ha-Ras) if treated with Olomoucine, Olomoucine II or Roscovitine at 32°C but not in any of the other rat fibroblasts. In contrast to 402/534 (young) and 602/534 (old) cells (both transfected with p53 only) which did not die in response to treatment with Roscovitine, Olomoucine or Olomoucine II on 32°C but remained arrested in G_1 during the whole period of cultivation on 32°C the cell line 173/1022 (isolated at day 15.5 gestation, expressing p53 and c-Ha-Ras) was even able to evade the initially detected G_1 arrest. Apparently the oncogene c-Ha-Ras acts as a save-guard preventing 173/1022 cells from p53 induced cell death. 189/111 cells isolated 2 days earlier obviously have not yet obtained this sort of 'protection'.

At 37°C where up to 70 % of p53 protein is mutated the differences between the four rat cell lines became even more apparent.

On 37°C all four rat cells arrest in G_2 evidenced by upregulation of $p21^{WAF1}$ protein and decrease in phosphorylation of Thr 160 of CDK. The cell cycle arrest was stronger in the young cell (day 13.5 gestation) lines 189/111 and 402/534 hence indicating that this cell cycle arrest was induced independent of c-Ha-Ras status but influenced by microenvironmental factors as older cells (day 15.5 gestation) are less influenced. Apparently there must be differences in environmental signalling leading to different responses of young and old cells to drug treatment.

The effector Caspases 3/7 were mainly activated in the two cell lines expression c-Ha-Ras probably due to p53 mediated pathways. Especially 189/111 cells turned out to be especially apoptosis prone in response to Olomoucine, Olomoucine II and Roscovitine. Our very preliminary data indicate that the differences in response of young and old c-Ha-Ras expressing cells are mediated by the Hedgehog Signalling pathway.

In contrast to cancer cells (HL-60 cells, HeLa cells, and rat transformed cell clones), the normal human MRC-5 lung fibroblasts and rat immortalized cells do not undergo apoptosis in response to treatment with Olomoucine, Olomoucine II or Roscovitine. This strongly substantiates the selectivity of the tested purine analogues and implicates that they are promising drugs in anti-cancer therapeutic strategy.

7. APPENDIX

7.1 Table of figures

7.2 List of Tables

7.3 References

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