

DIPLOMARBEIT

Outcome of treatment of human HPV-positive cervical cancer cells with selective inhibitors of CDKs

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Erst die Möglichkeit einen Traum zu
verwirklichen macht unser Leben lebens-
wert.

Paulo Coelho

TABLE OF CONTENTS

ABSTRACT	9
KURZZUSAMMENFASSUNG	11
1. INTRODUCTION	15
1.1. The eukaryotic cell cycle	15
1.1.1. Interphase	16
1.1.1.1. G ₁ phase	16
1.1.1.2. S phase	16
1.1.1.3. G ₂ phase	16
1.1.2. Mitosis	17
1.1.2.1. Prophase	17
1.1.2.2. Metaphase	17
1.1.2.3. Anaphase	17
1.1.2.4. Telophase	17
1.1.3. Cell cycle regulation	18
1.1.3.1. Cyclin-dependent kinases	18
1.1.3.2. Cyclins	19
1.1.3.3. CDK inhibitors	20
1.1.3.4. Cell cycle checkpoints	21
1.1.3.4.1. G ₁ restriction checkpoint	21
1.1.3.4.2. G ₁ /S checkpoint	22
1.1.3.4.3. intra-S checkpoint	22
1.1.3.4.4. G ₂ /M checkpoint	22
1.1.3.4.5. Spindle checkpoint	23
1.1.4. Molecular aspects of cell cycle progression	23
1.2. Programmed cell death	26
1.2.1. Cystein aspartic proteases (caspases)	26
1.2.2. Receptor-mediated pathway (extrinsic pathway)	27
1.2.3. Mitochondrial pathway (intrinsic pathway)	28
1.2.4. Regulation of apoptosis	28
1.2.4.1. FADD-like ICE inhibitory proteins (FLIPs)	29
1.2.4.2. Bcl-2 family	29
1.2.4.3. Inhibitors of apoptosis (IAPs)	29
1.3. Necrosis	30
1.4. Cancer	30
1.4.1. Characteristics of cancer	30
1.4.2. Malignant transformation leading to cancer	31
1.4.2.1. Proto-oncogenes	32
1.4.2.1.1. <i>Ras</i> oncogene	32
1.4.2.2. Tumor suppressor genes	33
1.4.2.2.1. Retinoblastoma protein (pRb)	33
1.4.2.2.2. Transcription factor p53	33
1.4.3. Other targets of cancer	35
1.5. Human papillomaviruses (HPV)	36
1.5.1. HPV genome structure	36
1.5.2. HPV life cycle	37
1.5.3. HPV E6 and E7 oncoproteins	39
1.5.3.1. E6 oncoprotein	39
1.5.3.2. E7 oncoprotein	40
1.5.4. Cervical cancer	41
1.5.4.1. Transmission and diagnosis	41
1.5.4.2. Symptoms and treatment	42
1.5.4.3. HPV vaccines	43

1.6.	Pharmacological CDK inhibitors as new therapeutic agents	43
1.7.	Aims of the present diploma thesis	44
2.	MATERIALS & METHODS	46
2.1.	Materials	46
2.1.1.	Cell lines	46
2.1.1.1.	HeLa S ₃	46
2.1.1.2.	HTB-31 (C-33A)	47
2.1.2.	Drugs	48
2.1.2.1.	Doxorubicin	48
2.1.2.2.	Olomoucine II	49
2.1.2.3.	Roscovitine	50
2.1.2.4.	Mimosine	50
2.1.2.5.	Trichostatin A	51
2.1.3.	Media	51
2.2.	Methods	52
2.2.1.	Cell culture	52
2.2.2.	Medications	53
2.2.3.	CellTiter-Glo [®] Luminescent Cell Viability Assay	54
2.2.4.	Measurement of the DNA content in single cells stained with propidium by flow cytometry	56
2.2.5.	Caspase-Glo [®] 9 Assay	56
2.2.6.	Apo-ONE [®] Homogeneous Caspase-3/7 Assay	58
2.2.7.	Caspase-Glo [®] 3/7 Assay	59
2.2.8.	7-Aminoactinomycin D dye exclusion test	59
2.2.9.	In situ M30 CytoDEATH staining	60
2.2.10.	DAPI staining	62
2.2.11.	Determination of the potential of mitochondrial membrane by JC-1 staining	63
2.2.12.	Whole cell lysates	64
2.2.13.	Cell fractionation – Isolation of nuclei	65
2.2.14.	Determination of protein concentration	66
2.2.15.	SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	67
2.2.16.	Electroblotting (Western Blot)	70
2.2.17.	Ponceau S staining	71
2.2.18.	Immunodetection	72
2.2.19.	Stripping of membranes	74
2.2.20.	Statistical evaluation	75
3.	RESULTS	78
3.1.	Effects of ROSC, OLO II and DOX on the proliferation of human HeLa and HTB-31 cervix carcinoma cells	78
3.1.1.	Anti-proliferative effects of ROSC in HeLa cells are time- and dose-dependent, the cell cycle status prior to the onset of treatment may influence the outcome	79
3.1.2.	Long-term effects of ROSC	80
3.1.3.	Higher ROSC concentrations are statistically highly significant	81
3.1.4.	OLO II is a more potent CDK inhibitor than ROSC	82
3.1.5.	Time course of OLO II action	82
3.1.6.	Anti-proliferative effects of ROSC and OLO II as compared to effects exerted by DOX, a highly cytotoxic drug	84
3.1.7.	HTB-31 cells are less susceptible to ROSC treatment	85
3.1.8.	OLO II affects HTB-31 cells more strongly than ROSC	86
3.1.9.	DOX treatment is not so effective in HTB-31 cells	88
3.2.	Effects of ROSC and OLO II on the cell cycle progression in HeLa and HTB-31 cancer cells	88
3.2.1.	ROSC induces a G ₂ /M arrest in HeLa cells accompanied by a high apoptosis rate	89
3.2.2.	OLO II arrests HeLa cells at G ₂ /M at low dosis	90

3.2.3.	ROSC rapidly arrests HTB-31 cells in G ₂ /M phase but pro-apoptotic effects are delayed	91
3.2.4.	Increase of the ratio of G ₂ /M cell population after OLO II treatment	93
3.3.	Modulation of the major cell cycle regulators upon treatment with selective CDK inhibitors	95
3.3.1.	ROSC prevents the activating phosphorylation of CDK2 and CDK1	95
3.3.2.	Repression of cdc25C phosphatase correlates with an accumulation of phosphorylation of CDK1 at inhibitory sites	97
3.3.3.	ROSC prevents pRb phosphorylation	98
3.3.4.	Inactivation of CDK7 mediated by ROSC	98
3.3.5.	ROSC and OLO II repress the function of RNA polymerase II in HeLa cells necessary for global transcription	99
3.3.6.	Increase of p53 levels upon ROSC treatment	100
3.3.7.	Repression of E6 and E7 viral oncoproteins in HeLa cells	101
3.3.8.	Site-specific phosphorylation of CDK7, but not that of CDK2 is affected in HTB-31 cells	103
3.3.9.	Transcriptional regulation in HTB-31 cells is affected by OLO II	104
3.3.10.	Neither ROSC nor OLO II affects p53 levels in HTB-31 cells	105
3.3.11.	ROSC induced upregulation of NF-κB in HTB-31 cells	105
3.4.	Apoptosis-promoting effects induced by the CDK inhibitors ROSC and OLO II in HeLa and HTB-31 cells	106
3.4.1.	Detection of caspase-3-cleaved cytokeratin 18 in cancer cells exposed to CDK inhibitors	107
3.4.2.	Loss of J-aggregate formation upon treatment of HeLa and HTB-31 cells indicate the collapse of the mitochondrial membrane	109
3.4.3.	Intracellular accumulation of 7-AAD as an evidence for the loss of the plasma membrane integrity	112
3.4.4.	ROSC and OLO II initiate caspase-dependent apoptosis in HeLa cells	114
3.4.5.	Phosphorylation of Bad and survivin contributes to ROSC-induced apoptosis	116
3.4.6.	Short-term ROSC treatment does not have any impact on apoptosis mediators in HTB-31 cells	117
3.4.7.	OLO II activates initiator and effector caspases in HTB-31 cells	119
3.5.	Synchronization of HeLa cells by mimosine and trichostatin A	120
3.5.1.	ROSC does not efficiently arrest cell cycle, when HeLa cells were previously synchronized by mimosine	121
3.5.2.	ROSC enhances G ₁ arrest in TSA-synchronized HeLa cells	122
3.5.3.	ROSC affects cell cycle and apoptosis regulators more efficiently, when HeLa cells were previously synchronized by TSA	125
4.	DISCUSSION	127
APPENDIX		131
I.	List of chemicals or other materials	131
II.	List of used antibodies	133
III.	List of Figures.....	135
IV.	List of Tables	139
V.	Abbreviations.....	140
REFERENCES		143
CURRICULUM VITAE		153
PUBLICATIONS		157

ABSTRACT

The application of pharmacological small-molecule inhibitors of cyclin-dependent kinases is a quite new and promising therapeutical strategy in combating cancer. Two inhibitors belonging to the group of purine analogues, namely roscovitine (ROSC) and olomoucine II (OLO II) seem to be of great clinical importance.

In this diploma thesis the action of both inhibitors on two different human cervix carcinoma cell lines was analyzed in detail. Considering the fact that ROSC and OLO II are structurally closely related – they differ in only one hydroxyl group – it was of interest to assess and to compare their effects on human HeLa and HTB-31 cervical cancer cells differing in the p53 and G₁/S checkpoint status. The question was raised whether and if yes, to what extent they would affect cellular proliferation, cell cycle progression and programmed cell death. In HeLa cells both tumor suppressor proteins, p53 and pRb, are not functional due to HPV infection, whereas in HTB-31 cells both tumor suppressor genes are mutated. In this context the issue appeared whether both inhibitors would be able to repress HPV-encoded proteins and in this way to reactivate p53 and restore the checkpoint in HeLa cells.

Our results demonstrated the high anti-proliferative effects of ROSC and OLO II. Both inhibitors reduced the numbers of living cells in a time- and concentration dependent manner. The reduction of cell numbers was attributable to induction of cell cycle arrest and/or apoptosis. However, the outcome strongly depends on drug concentration. ROSC at lower dosage (20 μ M) induced a G₂/M arrest that in HTB-31 cells appeared with some delay. Higher concentrations of the same drug induced caspase-dependent apoptosis primarily in HeLa cells, as evidenced by activation of caspase-3. The increase of caspase-3 coincided with changes in a balance between pro- and anti-apoptotic factors: the upregulation of the pro-apoptotic protein Bad and the concomitant inactivation of survivin. Unlike HeLa cells, HTB-31 cells were more resistant to ROSC action and apoptotic cells appeared after a longer treatment time (48h or longer). These observations are not surprising because p53 and pRb tumor suppressor proteins are mutated and CDK inhibitors are not able to reactivate them.

Interestingly, OLO II was more efficient than ROSC in both cell lines, exerting exactly the same effects, however at lower doses.

Moreover, the importance of the status of cancer cells prior to the onset of treatment for the outcome of therapy was examined. Asynchronously growing cells and synchronized cells

were exposed to drugs. Medication of asynchronously growing cells was started at different time points after cell plating. Cells after a short recovery period (10h) were G₁-arrested and entered the active cell cycle after a longer period of time (22h) after plating. Interestingly, cells after a short recovery period were less sensitive to drugs than cells at 22h post-plating. Additionally, synchronized cells released from the block were also exposed to CDK inhibitors. ROSC was able to prolong cell cycle arrest of synchronized HeLa cells only in early but not in late G₁ phase.

Analysis of cellular proteins in samples obtained from untreated controls and cells treated with both CDK inhibitors for increasing periods of time by immunoblots provided details about the mechanism of the action of ROSC and OLO II.

Both inhibitors abrogated activation of CDK7 and in this way were able to repress the site-specific phosphorylation of CTD of RNA Polymerase II resulting in the block of overall transcription in HeLa cells. Transcriptional breakdown led to downregulation of E6 and E7 oncogenic proteins in HeLa cells. In untreated HeLa cells these virally encoded proteins bind and inactivate p53 and pRb, respectively, thereby contributing to malignant transformation. Hence, abrogation of E6 and E7 reconstituted the G₁ restriction checkpoint, evidenced by the reactivation of pRb protein and the strong p53 upregulation after ROSC treatment. Restored p53 activity contributes to cell cycle arrest and apoptosis and represents a further mechanism of ROSC action. HTB-31 cells in contrast were not affected in p53 levels, indicating that CDK inhibition is not sufficient to counteract the mutated protein. Additionally, synchronization of HeLa cells with trichostatin A, a deacetylase inhibitor, before the actual ROSC treatment revealed improved effects of the latter in comparison to a treatment of unsynchronized cells.

Taken together, ROSC and OLO II display high therapeutic potential due to their increased selectivity towards transformed cells. Their ability to modulate the cell cycle and to induce a controlled cell death additionally is associated with relatively low, if any, cytotoxicity. The weak cytotoxicity of CDK inhibitors is a great advantage. Moreover, the pleiotropic effects exerted by ROSC and OLO II are of advantage too.

KURZZUSAMMENFASSUNG

Die Anwendung von pharmakologischen Cyclin-abhängigen Kinase Inhibitoren ist eine ziemlich neue und vielversprechende therapeutische Strategie in der Bekämpfung von Krebs. Zwei Inhibitoren, die zur Gruppe der Purine Analoge zählen, nämlich Roscovitin (ROSC) und Olomoucine II (OLO II), scheinen von großer klinischer Bedeutung zu sein.

In dieser Diplomarbeit wurden die Effekte beider Inhibitoren auf zwei verschiedene humane Zervix Karzinom Zelllinien im Detail untersucht. Zieht man die Tatsache in Betracht, dass ROSC und OLO II in ihrer Struktur sehr ähnlich sind – sie unterscheiden sich in nur einer Hydroxylgruppe voneinander – war es von Interesse ihre Wirkung auf humane HeLa und HTB-31 Zervix Krebszellen, die sich im p53 und G₁/S Checkpoint Status unterscheiden, zu bestimmen und untereinander zu vergleichen. Die Frage kam auf, ob und wenn ja, in welchem Ausmaß beide Inhibitoren die zelluläre Proliferation, die Zellzyklusprogression und den programmierten Zelltod beeinflussen würden. In HeLa Zellen sind beide Tumorsuppressor Proteine, p53 und pRb, auf Grund einer HPV Infektion nicht funktionell, wobei in HTB-31 Zellen beide Tumorsuppressor Gene mutiert sind. In diesem Zusammenhang tauchte die Frage auf, ob beide Inhibitoren im Stande sein würden die HPV-kodierten Proteine zu reprimieren und dadurch p53 zu reaktivieren und den Checkpoint in HeLa Zellen wiederherzustellen.

Unsere Resultate zeigen die starken anti-proliferativen Effekte von ROSC und OLO II. Beide Inhibitoren reduzierten die Zahl der lebenden Zellen in einer Zeit- und Konzentrations-abhängigen Art und Weise. Die Abnahme der Zellzahl ist auf die Induktion eines Zellzyklusarrests und/oder Apoptose zurückzuführen. Das Ergebnis hängt jedoch stark von der Medikamentenkonzentration ab. ROSC in einer niedrigeren Dosierung (20 µM) induzierte einen G₂/M Arrest, welcher in HTB-31 mit einiger Verzögerung auftrat. Höhere Konzentrationen desselben Medikaments verursachten eine Caspase-abhängige Apoptose primär in HeLa Zellen, die durch die Aktivierung der Caspase-3 nachgewiesen wurde. Die Zunahme von Caspase-3 stand in Einklang mit den Veränderungen in der Balance zwischen pro- und anti-apoptotischen Faktoren: die erhöhte Expression des pro-apoptotischen Proteins Bad und die zeitgleiche Inaktivierung von Survivin. Im Gegensatz zu HeLa Zellen waren HTB-31 Zellen gegenüber der Wirkung von ROSC resistenter und apoptotische Zellen traten erst nach einer längeren Behandlungszeit auf (48h oder länger). Diese Beobachtungen sind nicht überraschend, da die p53 und pRb Tumorsuppressor Proteine mutiert sind und CDK Inhibitoren nicht fähig sind sie zu reaktivieren.

Interessanterweise war OLO II in beiden Zelllinien effizienter als ROSC, wobei es dieselbe Wirkung, allerdings bei niedrigeren Dosen, ausübte.

Weiters wurde die Bedeutung des Zustandes von Krebszellen vor dem Beginn der Behandlung für den Ausgang der Therapie untersucht. Asynchron wachsende und synchronisierte Zellen wurden den Medikamenten ausgesetzt. Die Verabreichung der Medikamente wurde im Fall der asynchron wachsenden Zellen nach unterschiedlichen Zeitpunkten nach dem Ansetzen vorgenommen. Zellen, die eine kurze Erholungszeit (10h) hatten, wurden in der G₁ Phase arretiert und betraten den aktiven Zellzyklus nach einer längeren Zeit (22h) nach dem Ansetzen. Interessanterweise waren Zellen nach einer kurzen Erholungszeit weniger empfindlich gegenüber den Medikamenten als Zellen nach 22h nach dem Ansetzen. Zusätzlich wurden synchronisierte Zellen, die aus dem Arrest entlassen wurden, verschiedenen CDK Inhibitoren ausgesetzt. ROSC war im Stande den Zellzyklusarrest von synchronisierten HeLa Zellen in der frühen, nicht aber in der späten, G₁ Phase zu verlängern.

Die Analyse der zellulären Proteine in Proben von unbehandelten und mit beiden CDK Inhibitoren behandelten Zellen über zunehmende Zeitspannen durch Immunoblots gaben Einsicht in die Mechanismen der ROSC und OLO II Wirkung.

Beide Inhibitoren unterdrückten die Aktivierung von CDK7 und waren dadurch im Stande die ortsspezifische Phosphorylierung der CTD der RNA Polymerase II zu hemmen. Daraus resultierte ein Block der allgemeinen Transkription. Der Zusammenbruch der Transkription führte zur verminderten Expression der E6 und E7 onkogenen Proteine in HeLa Zellen. In unbehandelten HeLa Zellen binden und inaktivieren diese viral kodierten Proteine p53 beziehungsweise pRb und tragen dadurch zur malignen Transformation bei. Demzufolge stellte die Außerkraftsetzung von E6 und E7 den G₁ Restriktionscheckpoint wieder her. Dies wurde durch die Reaktivierung des pRb Proteins und der starken Hochregulation von p53 nach einer ROSC Behandlung nachgewiesen. Die wiederhergestellte p53 Aktivität trägt zum Zellzyklusarrest und zur Apoptose bei und repräsentiert dabei ein weiterer Mechanismus der ROSC Wirkung.

Im Gegensatz dazu wurden die p53 Mengen in HTB-31 Zellen nicht beeinflusst. Das weist daraufhin, dass die Hemmung von CDKs nicht ausreichend ist um dem mutierten Protein entgegenzuwirken. Zusätzlich zeigte die Synchronisierung von HeLa Zellen mit Trichostatin A, ein Deacetylase Inhibitor, vor der eigentlichen ROSC Behandlung die verbesserte Wirkung von ROSC im Vergleich zur Behandlung von nicht synchronisierten Zellen.

Insgesamt weisen ROSC und OLO II ein hohes therapeutisches Potential auf Grund ihrer erhöhten Selektivität gegenüber transformierten Zellen auf. Ihre Fähigkeit den Zellzyklus

zu modulieren und zusätzlich einen kontrollierten Zelltod zu induzieren hängt mit ihrer relativ niedrigen, wenn überhaupt vorhandenen Zytotoxizität zusammen. Die geringe Zytotoxizität von CDK Inhibitoren ist ein großer Vorteil ebendieser. Außerdem sind die pleiotropen Effekte, die von ROSC und OLO II ausgeübt werden, ebenfalls ein großer Vorteil.

1. INTRODUCTION

Cancer is one of the most common diseases mankind is confronted with. According to the American Cancer Society cancer is the second leading cause of death in the United States. Nearly everybody has or was indirectly confronted with this disease. ^[1] In Austria nearly 25 % of carcinoma patients die finally. ^[2]

Due to the fact that cancer is a process creeping over years until fully developed, it can remain long time undiscovered and displays then severe effects. It is quite scaring that everyone of us can develop cancer, but the most striking certainty is that 30 % of all cancer death can be prevented by simple lifestyle changes. ^[3]

Cancer is a disease not easy to cure, especially when it is not detected in early stages. This is because of the sophisticated and subtle network cells display and cancer targets. Besides, tumorigenic cells have the ability to spread throughout the body forming metastasis in late stages of development and even if cancer is treated, reoccurrence is very likely. However, mortality seemed to decline in the last few years, a success attributed to treatment therapies. Nevertheless, up to the present anti-cancer therapies like radiotherapy, chemotherapy with cytostatics or even surgery are not highly selective and may exert various side effects as cytotoxicity towards healthy cells. Hence, they are associated with severe undesired complications. Therefore the interest in appropriate treatments is great.

Recently discovered pharmacological cyclin-dependent kinase inhibitors seem to be a promising novel therapy in combating cancer. This diploma thesis will deal with this new form of treatment analyzing their effects on a special type of cancer cells maintained in culture.

But to start from the beginning, basic cellular proliferation and its regulation mechanisms have to be understood first in order to understand cancer. They will be depicted in detail in the next sections.

1.1. The eukaryotic cell cycle

The eukaryotic cell cycle is an essential process in every cell's life which ensures its proper proliferation including correct DNA replication and cell division. The ultimate result is the generation of two identical daughter cells. ^[4]

The cell cycle is divided into a mitosis phase (M phase) and an interphase, each subdivided into more phases fulfilling different functions (Fig. 1). ^[5]

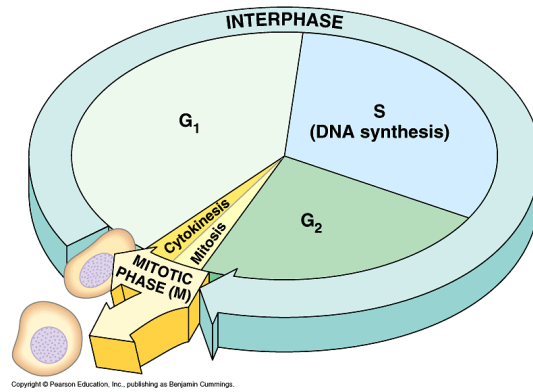


Fig. 1: Schematic view of cell cycle stages ^[6]

1.1.1. Interphase

The interphase is the phase between two sequential mitosis representing 95 % of the mammalian cell cycle. During interphase chromosomes are decondensed, cells start growing doubling their size and DNA replication proceeds. In other terms cells prepare for the next cell division round. ^[5]

Three distinct phases can be distinguished in interphase:

1.1.1.1. G₁ phase

The gap phase 1 is the phase between mitosis and S phase. Cells in G₁ phase are metabolically active; they synthesize RNA and proteins ^[7] and start to grow. Thus they prepare for the upcoming DNA synthesis. ^[5] At this stage cells are responsive for growth signals ^[4], nutrients or external/internal inhibitors influencing a cell's decision to enter or to leave the cell cycle. ^[8] Once cells cross the G₁ restriction checkpoint in late G₁ phase the decision for cell cycling is irreversible.

1.1.1.2. S phase

During the synthesis phase a cell replicates its DNA, while RNA and protein synthesis are maintained. ^[7] Specific regulation mechanisms assure that chromosomes are duplicated only once per cell cycle. Therefore cells are committed to proceed to M phase before beginning a new replication round (though a few exceptions exist). ^[9] At the end of this phase a twofold chromosome set is present.

1.1.1.3. G₂ phase

The gap phase 2 is a postsynthetic phase, where cell growth reaches a maximum. RNA and proteins important for the sequential mitosis are synthesised. At this stage specific control

mechanisms proof fidelity of DNA replication, size of cells and energy supply necessary to proceed to M phase. ^[7]

1.1.2. Mitosis

The mitosis phase is the shortest phase but probably the most spectacular one. ^[5] Cells use their whole energy for the nuclear division with a subsequent cytokinesis. Hence, protein synthesis continues at a low rate and RNA synthesis takes only place in early prophase and late telophase. ^[7]

During mitosis the DNA is condensed to chromosomes and changes in the rearrangement of mitotic spindles, cytoskeleton and nuclear envelope occur. Thus a faithful segregation of the chromosomes is facilitated. ^[8] Mitosis leads to a reduction of tetraploidy resulting in two diploid cells. ^[7]

Mitosis includes four distinct phases:

1.1.2.1. Prophase

During prophase the DNA is condensed to chromosomes in order to enable the following separation, whereas sister chromatids are only kept together at centromeres. The centrosomes move to the opposite poles and the mitotic spindle starts to develop. Finally the nuclear envelope is disintegrated. ^[5]

1.1.2.2. Metaphase

Microtubules attach to chromosome kinetochores and link centrosomes to the condensed DNA. Due to microtubule movement (by pulling or pushing) chromosomes are aligned at the metaphase plate and are ready for segregation. ^[5]

1.1.2.3. Anaphase

The first event in anaphase is the separation of sister chromatids at the kinetochor. Then microtubule contraction pulls the chromatids to opposite poles of the mitotic spindle. ^[5]

1.1.2.4. Telophase

In telophase nuclei start to reform around each mitotic spindle and chromosomes decondense again. ^[5]

To complete cell division, cytokinesis takes place. Cytokinesis is the division of the cytoplasm during late anaphase and telophase. It is accomplished by a contractile ring com-

posed of myosin II and actin filaments. As a consequence constriction of the cell membrane occurs. The position of ring formation is dependent on the position of the mitotic spindle. ^[5]

However, not all eukaryotic cells participate in the cell cycle. Some cells do not proliferate or grow anymore. They exit the active cell cycle and remain in a quiescent stable state, the **G₀ phase**. ^[10] Either they have reached their final developmental state and do not need to proliferate any more, e.g. neuronal or muscle cells, or different environmental factors influence them to leave the cell cycle. ^[11] Quiescence is lifelong or reversible after undefined periods of time. Mitogenic signals can reactivate cells, but re-entry into G₁ phase of cell cycle needs continuous stimulation until the G₁ restriction checkpoint is overwhelmed. ^[12]

1.1.3. Cell cycle regulation

Evidently the cell cycle is a temporal and sequential restricted process which has to be tightly regulated. Thus progression through different cell cycle stages is coordinated by different proteins. The main positive regulators are heterodimeric serine/threonine kinase complexes each composed of a catalytic cyclin-dependent kinase (CDK) and a regulatory cyclin (cyc) subunit. In contrast inhibitory proteins of the Cip/Kip or INK4 family influence indirectly the cell cycle. ^[13]

1.1.3.1. Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) have different functions in cellular proliferation and RNA transcription. The most CDKs supervise cell cycle stages (e.g. CDK1, 2, 4), whereas transcriptional CDKs (e.g. CDK7, 8, 9) phosphorylate the carboxy-terminal domain of RNA polymerase II. Thus transcriptional activation takes place. ^[14] Up to now 11 different CDKs and 9 CDK-like proteins have been discovered. ^[15]

CDKs are expressed during the cell cycle at constant levels as inactive proteins and underlie a two-step activation process involving several other proteins. Their association with cyclins leads to a partial activity, whereas postsynthetic modification in the conserved T-loop (e.g. phosphorylation, acetylation) triggers full activity. This is accomplished by the CDK-activating kinase (CAK), being a CDK-cyclin complex itself. ^[13] Additionally inhibitory phosphorylation at threonine and tyrosine residues by Wee1/Myt1 kinases can occur. Cdc25 phosphatases reverse the process. If CDKs are phosphorylated at inhibitory and activating residues, the whole protein is inactive.

The interaction of CDKs with CDK inhibitors leads to their inactivation thus modulating the cell cycle. Finally CDK activity is regulated by protein folding and subcellular localization. ^[15]

Figure 2 summarizes the different regulation mechanisms acting on CDKs.

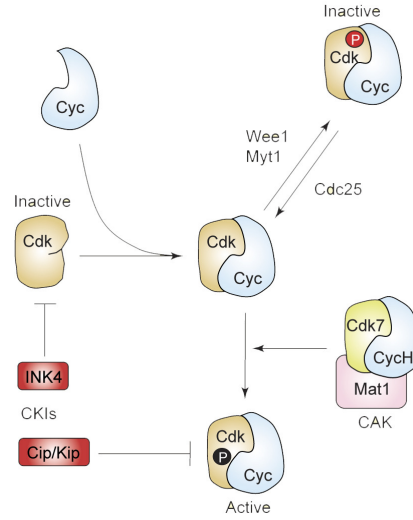


Fig. 2: Regulation mechanisms of CDKs ^[15]

1.1.3.2. Cyclins

As regulatory subunits cyclins induce conformational changes in CDKs, thus activating them. ^[16] However, they exhibit a nuclear localization signal (NLS) as well, so that CDKs are directed to the nucleus to accomplish their role in cell cycle. ^[17]

The expression of cyclins oscillates during the cell cycle (Fig. 3). At defined points of time distinct cyclin amounts slightly increase until they reach a certain threshold level. This triggers the activation process of CDKs and indicates the passage of a cell cycle stage. After fulfilling their mission, cyclins decline abruptly. ^[18] Hence, cyclins are regulated due to a balance between *de novo* synthesis and targeted degradation. Consequently CDK activation occurs in a time-dependent manner, whereas the availability of different cyclins determines the type of CDK activated and the type of substrate targeted. ^[19] Cyclin regulation may also be achieved by phosphorylation or subcellular localization.

Proteolytic degradation of cyclins is a multi-step process which involves site-specific phosphorylation creating phosphodegron, polyubiquitin tagging of proteins mediated by specific E3 ubiquitin ligase, nuclear export and subsequent degradation in proteasome. ^[20]

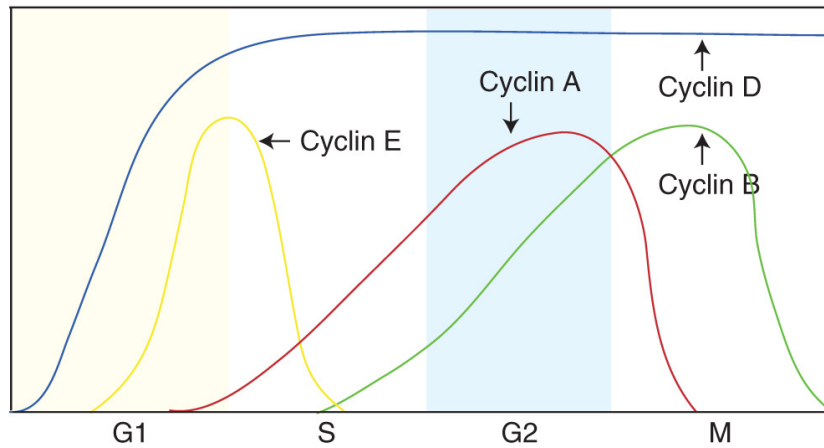


Fig. 3: Cyclin expression during cell cycle ^[21]

The purpose of activated CDK-cyclin complexes is the phosphorylation of target proteins at specific serine or threonine residues to coordinate progression through or entry into the next cell cycle phase. Such target proteins are among others the tumorsuppressor protein pRb, Wee1 kinase, cdc25 phosphatase, cytoskeletal proteins or microtubules. ^[10]

1.1.3.3. CDK inhibitors

As mentioned above CDK inhibitors counteract CDK-cyclin activity and are important regulation processes of healthy cells. Anti-proliferative signals (e.g. DNA damage, limiting nutrients etc.) or intracellular factors directly activate CDK inhibitors to stop cell cycle progression and to direct cells to a quiescent state. ^[21]

CDK inhibitors can be subdivided into two classes: the Cip/Kip protein family (e.g. p21^{Cip1/Waf1}, p27^{Kip1}, p57^{Kip2}) and the INK4 proteins (inhibitors of CDK4, e.g. p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, p19^{INK4d}). ^[22]

The Cip/Kip family members bind to active CDK-cyclin complexes to inhibit both of them. It was documented that Cip/Kip inhibitors bind exactly to the ATP binding site in CDKs. Thus insertion into the catalytic cleft blocks ATP binding and CDK's catalytical activity. ^[13] Additionally, Cip/Kip proteins have also different functions in apoptosis, transcriptional regulation, cell fate determination and cell migration. ^[23]

INK4 proteins in contrast are specific for G₁ phase CDKs, that means CDK4 and 6, and bind to their monomeric form. CDK activation by cyclins is not inhibited by blocking the cyclin binding site, but results from allosteric changes inside the very same position. Thus they prevent the activation by cyclins. Although it was also reported that INK4 inhibitors deform the ATP binding site. ^[13]

1.1.3.4. Cell cycle checkpoints

Cell cycle checkpoints are quality control points between cell cycle phases. They provide opportunity for cells to interrupt the cell cycle and to carefully examine if physiological conditions permit a further progression. Physiological conditions refer to certain external factors (e.g. nutrients, growth factors, cell density, presence of DNA damaging agents) and/or internal factors (e.g. DNA content and integrity) which exert influence on cells. Once a checkpoint is traversed, return to the previous stage is irreversible. ^[24]

Each checkpoint disposes of a sensor mechanism to recognize aberrant or incomplete cell cycle events and a signal transduction pathway to transfer the signal to effector proteins. As a consequence measures are taken to reconstitute the proper cell cycle. ^[4] Cell cycle checkpoints themselves are mainly controlled by CDK inhibitors and CDK-cyclin complexes.

The main checkpoints are outlined in Figure 4 and described in more detail underneath.

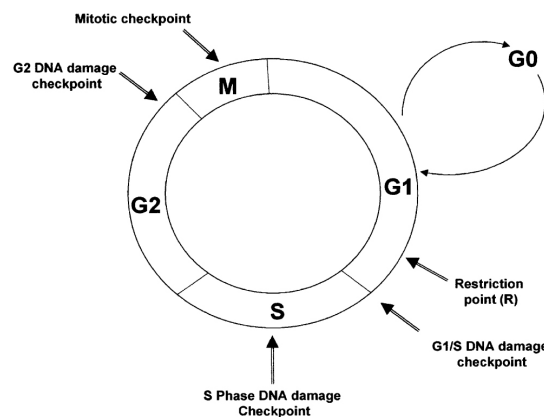


Fig. 4: Cell cycle checkpoints restricted to defined spots ^[4]

1.1.3.4.1. G₁ restriction checkpoint

The G₁ restriction checkpoint in the middle/late G₁ phase permits cells to assure themselves if nutrients and proliferating signals are sufficient to be committed to DNA replication. Proliferative signals are required until the restriction checkpoint is passed. If this is not the case, cells return to a quiescent state leaving the cell cycle. ^[4] The restriction checkpoint is controlled by the retinoblastoma tumor suppressor protein (pRb). ^[17] During G₁ phase hypophosphorylated pRb binds and inactivates consequently the E2F transcription factor, which is important for S phase genes. As cells progress in cell cycle CDK4 and CDK6 as well as cyclin D are synthesized. Active complexes composed of CDK4-cyclinD or CDK6-cyclinD phosphorylate pRb at three critical residues. However, only the phos-

phorylation at a fourth serine residue by the active CDK2-cyclinE complex leads to induction of S phase genes by releasing E2F. The restriction checkpoint is passed. ^[25]

1.1.3.4.2. G₁/S checkpoint

DNA damage of endogenous or exogenous source induces G₁/S, intra-S and G₂/M checkpoints. ^[26]

Depending on type of DNA lesions responsive ATM/ATR and downstream Chk1/Chk2 kinases are activated. This leads to phosphorylation of the tumor suppressor and transcription factor p53, which induces consequently p21^{Cip1/Waf1} expression. ^[27] p21^{Cip1/Waf1} is known to inhibit G₁ kinases. ^[17] However it represents a slow mechanism of counteracting. A much more rapid way constitutes the downregulation of cdc25A phosphatase thus inhibiting important CDK-cyclin complexes (CDK2-cyclinE and CDK2-cyclinA). ^[27]

1.1.3.4.3. intra-S checkpoint

The intra-S checkpoint is an exception forasmuch as it only results in a temporally delay of cell cycle progression. Presumably two parallel mechanisms are involved in the inhibition of the replication initiation at different sites. One of them coincides with the ATM-phosphatase cdc25 pathway described above, the other one includes ATM activation and phosphorylation of NBS1, a DNA repair protein. ^[27] The overall result is a slower DNA replication thus lengthening the S phase. ^[4] Nevertheless far too few knowledge exists about this checkpoint.

1.1.3.4.4. G₂/M checkpoint

Upon DNA damage the same components as in G₁/S checkpoint are activated in G₂ phase (referring to ATM/ATR and checkpoint kinases Chk 1/2), however, their specific downstream targets differ. The main purpose of G₂ phase arrest upon DNA injury is the inhibition of the CDK1-cyclinB mitosis promoting factor. Checkpoint kinases phosphorylate the cdc25C phosphatase at Ser²¹⁶ thus promoting its association with 14-3-3 proteins and sequestration in the cytoplasm. Under favourable conditions active cdc25C would normally dephosphorylate the CDK1-cyclin B complex at the inhibitory residues threonine¹⁴ and tyrosine¹⁵ to permit a progression to mitosis phase. Since CDK1-cyclinB complexes are in the nucleus, cdc25C phosphatase is blocked and a G₂ arrest is induced.

Additionally p53 upregulation can indirectly block kinase activity via p21^{Cip1/Waf1} and induce 14-3-3- σ protein expression. 14-3-3- σ binds to CDK1-cyclinB complexes and retains them in the cytoplasm far away from the nucleus. ^[4]

1.1.3.4.5. Spindle checkpoint

The spindle checkpoint resides in metaphase/anaphase of mitosis and assures a correct chromosome segregation to prevent cell aneuploidies. It is triggered if the mitotic spindle has not formed or not attached properly to sister chromatids.

During mitosis segregation of chromatids is a coordinated process that takes not place until all chromosomes are arranged at the equatorial plate and attached to microtubules. Shortly after the APC (anaphase-promoting complex) E3 ligase associates with cdc20 and is phosphorylated by CDK1-cyclinB to become active. APC is indispensable for chromosomal segregation since it targets the protein securin for ubiquitin mediated degradation. This releases the protease separase which in turn cleaves the cohesine rings that hold sister chromatids together. Separation of sister chromatids is now feasible.

Proteins involved in activation of the spindle checkpoints are Mad1/2 (mitotic arrest-deficient) and Bub1 (budding uninhibited by benzimidazole) proteins. They bind to loosen kinetochores of chromatids and inhibit the APC complex (by binding to its regulatory subunit cdc20) thus blocking securin degradation and chromatid segregation. ^[4]

The overall outcome of activated cell cycle checkpoints is an induced cell cycle arrest with a possible DNA repair or in the worst case an induced programmed cell death (apoptosis). ^[28]

1.1.4. Molecular aspects of cell cycle progression

Somatic cells are capable to react to environmental stimuli as well as to intracellular signals and to correctly link the response to these. The molecular mechanisms behind are outlined in the following detailed view.

Somatic cell division is initiated by a receptor mediated signalling pathway, called the MAPK (mitogen-activated protein kinase) signalling pathway (see Fig. 5). It is a conserved mechanism which controls cellular growth, proliferation, differentiation and survival of cells. Due to stimulation (e.g. growth factors, stress, hormones etc.) of receptor tyrosine kinases in the cellular plasma membrane small GTP-binding molecules consequently activate three downstream kinases. These kinases, termed MAPKKK (MAPKK kinase), MAPKK (MAPK kinase) and MAPK sequentially phosphorylate each other: MAPKKK

phosphorylates and activates MAPKK, MAPKK phosphorylates and activates MAPK. [29] Thus activated MAP kinase phosphorylates and activates transcription factors or other serine/threonine kinases which induce specific cellular responses. [30]

Three distinct MAPK signal transductions are well-known, the Erk, Jnk and p38 MAPK pathway, whereas the Raf-MEK1/2-ERK1/2 pathway is the most frequent one (Fig. 5). Although activation of all three pathways follows the same principle, the type of stimulation and outcome differ. [30]

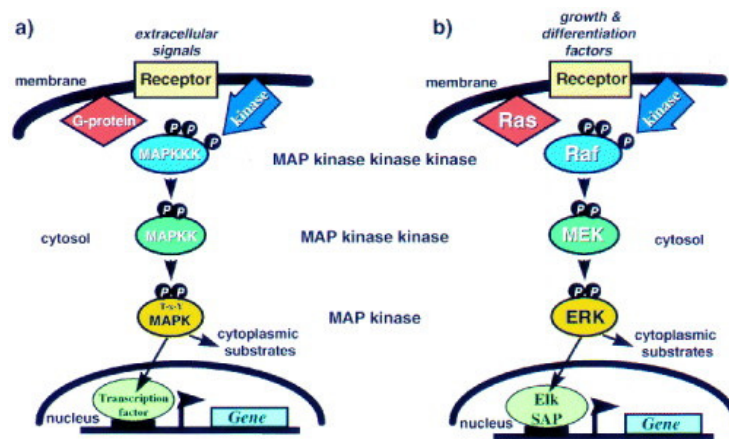


Fig. 5: **a.** General MAPK signal transduction pathway step by step, **b.** Raf-MEK1/2-ERK1/2 pathway in detail [31]

So in response to extracellular signals cells activate the Raf-MEK1/2-ERK1/2 pathway to synthesize type D cyclins (cyclin D1, D2 and D3) and thus to turn on cell cycle. [22] Cyclin D forms complexes with CDK4/6 travelling that way to the nucleus to become fully activated by CAK kinase. Thereby the type of cyclin D seems not to be relevant. However due to the short-lived properties of D-type cyclins it is of great importance that cells receive continuously mitogenic signals for a permanent cyclin D synthesis. Otherwise the cell cycle would be interrupted and cells enter quiescence. [32]

Active CDK4-cyclinD or CDK6-cyclinD complexes phosphorylate the tumor suppressor protein pRb at three critical residues (Ser⁷⁸⁰ and Ser^{807/811}) in order to inactivate it during G₁ phase. At the beginning of G₁ phase pRb is hypophosphorylated (or not phosphorylated at all) and tightly bound to the transcription factor E2F, which is important for S phase gene expression. [4] pRb masks the transactivation domain of E2F and keeps it inactive by that way. Additionally it interacts with histone deacetylases to repress premature transcription. [33] As soon as pRb phosphorylation occurs, cyclin E expression is induced. [34] Association of cyclin E with CDK2 in late G₁ phase results in pRb phosphorylation (at the fourth serine⁷⁹⁵) and ultimate lost in its growth-inhibitory power. [19] This is the moment

when a cell leaves the restriction checkpoint behind and is irreversibly committed to DNA replication and cell division (thus not responsive to extracellular signals anymore).^[15] As a consequence the E2F transcription factor is released from Rb binding and becomes transcriptionally active.^[4] Cells proceed to S phase where E2F initiates transcription of important genes like cyclin A.^[35] At the same time cyclin E is proteolytically degraded to ensure just one replication round per cell division.^[15]

Progression through S phase is driven by active CDK2-cyclinA kinases which target proteins specific for DNA replication. Amongst others Rb hyperphosphorylation is kept up until mitosis completion.^[22] Towards the end of S phase cyclin A rapidly complexes with CDK1, whereas CDK1 seems to be poorly phosphorylated at inhibitory residues.^[36] Consequently E2F promoted transcription is repressed, resulting in a repressed E2F accumulation and S phase exit.^[37] During G₂ phase cyclin A-CDK1 kinase enhance activation of cyclin B associated CDK1 complexes by inhibiting the kinase responsible for inhibitory phosphorylation on CDK1.^[36] Cyclin B-CDK1 complexes regulate the transition through the G₂/M checkpoint and the mitosis phase.^[15] Initiation of mitosis requires phosphorylation of CDK1 at threonine¹⁶¹ and removal of phosphate groups from the inhibitory sites by cdc25C phosphatase.^[8] Cyclin B associated CDK1 kinases influence then several ongoing processes during mitosis, like chromosomal condensation, centrosome separation, breakdown of nuclear lamina and guide through mitotic progression.^[15]

Finally, an appropriate exit from mitosis necessitates inactivation of CDK1-cyclinB complexes, which is performed by APC during metaphase.^[15] APC ubiquitinates cyclin B, thus it triggers its proteosomal degradation. Concomitantly, CDK1 undergoes a conformational change which leads to its complete inactivation.^[38]

Figure 6 outlines CDK-cyclin complexes important in cell cycle progression.

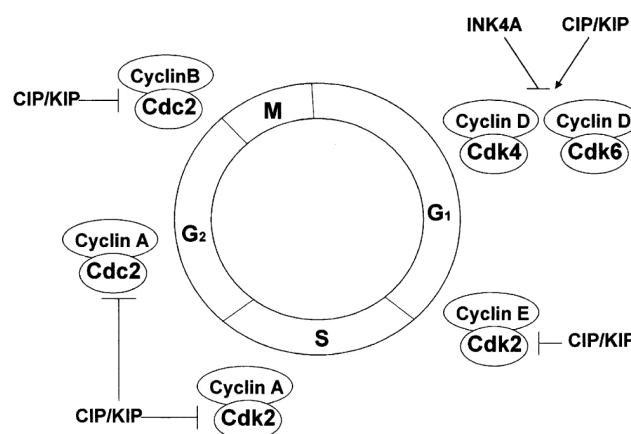


Fig. 6: Cyclin-CDK complexes responsible for cell cycle progression^[4]

1.2. Programmed cell death

Together with cellular proliferation organisms harbour a mechanism for programmed cell death (apoptosis). To keep the balance between both processes is indispensable to life. Disorders in both events may lead to autoimmunity, cancer and other diseases. ^[39]

Apoptosis is a physiological process of natural cell suicide in order to eliminate damaged, dangerous or a surplus of cells. ^[40] It is an active, controlled process which in contrast to necrosis (also believed to be a form of cell death) does not trigger any inflammatory response. Supplementary it does not cause any harm to the organism. ^[41] Apoptosis plays an immense role in tissue homeostasis, defense against pathogens, elimination of unnecessary cells and generally in the development of organisms. Typical, common morphological features are cell shrinkage, chromatin and cytoplasm compaction and DNA fragmentation, ^[40] all ending in the generation of apoptotic bodies which are then removed by phagocytosis. Apoptotic bodies are fragments/vesicles surrounded by intact membranes to prevent leakage of cellular contents, which otherwise would trigger an immune response. ^[42]

There are two alternative pathways of apoptosis induction, the mitochondrial and the receptor-mediated pathway. For both pathways caspases (cysteine aspartic proteases) are important mediators ^[39], which lead to a complete degradation of cellular components. ^[43]

1.2.1. Cystein aspartic proteases (caspases)

Caspases are proteases stimulated by pro-apoptotic signals to induce a downstream caspase cleavage cascade ending in degradation of the cell. These proteases are synthesized as inactive precursors (pro-caspases) and proteolytically cleaved to active enzymes (by themselves or by other proteases), whereas once activated no return is possible. ^[44] Caspases are usually made up of a large and a small subunit linked to a pro-domain. After proteolysis (at aspartat residues) the prodomain is displaced and caspases form heterodimers with other caspases, in which the small subunits face each other (Fig. 7). ^[45]

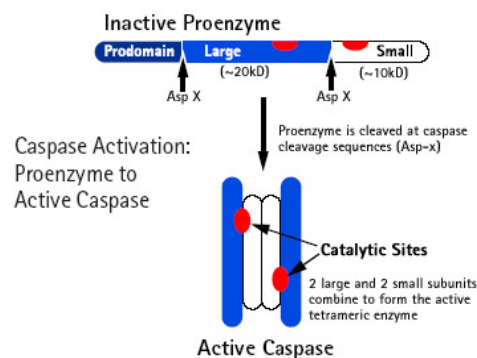


Fig. 7: Caspase zymogens forming heterodimers when activated ^[46]

Two families of caspases can be distinguished, namely the inflammatory and the apoptotic caspases, whereas the latter include initiator and effector caspases. Initiator caspases (caspase-2, -8, -9 and -10) are recruited in multiplicity via adaptor proteins and auto-activated due to close vicinity. These caspases initiate the caspase cascade leading to the activation of downstream effector caspases. Effector caspases (caspase-3, -6 and -7) cleave cellular proteins thus dismantling cells. ^[47]

1.2.2. Receptor-mediated pathway (extrinsic pathway)

This apoptosis pathway is triggered when corresponding ligands (e.g. FAS – a transmembrane protein) bind to specific cell surface receptors belonging to the tumor necrosis factor (TNF) family due to death stimuli (Fig. 8). ^[48] TNF receptors trimerize upon activation, ^[49] thus recruiting the adaptor protein FADD (Fas-associated death domain protein) to their cytoplasmic death domain. FADD binds then the initiator caspases, pro-caspase-8 and pro-caspase-10, to form the death-inducing signalling complex (DISC). ^[39] The result is auto-proteolytic cleavage of caspase-8/10 and activation of downstream effector caspases (e.g. caspase-3). ^[49]

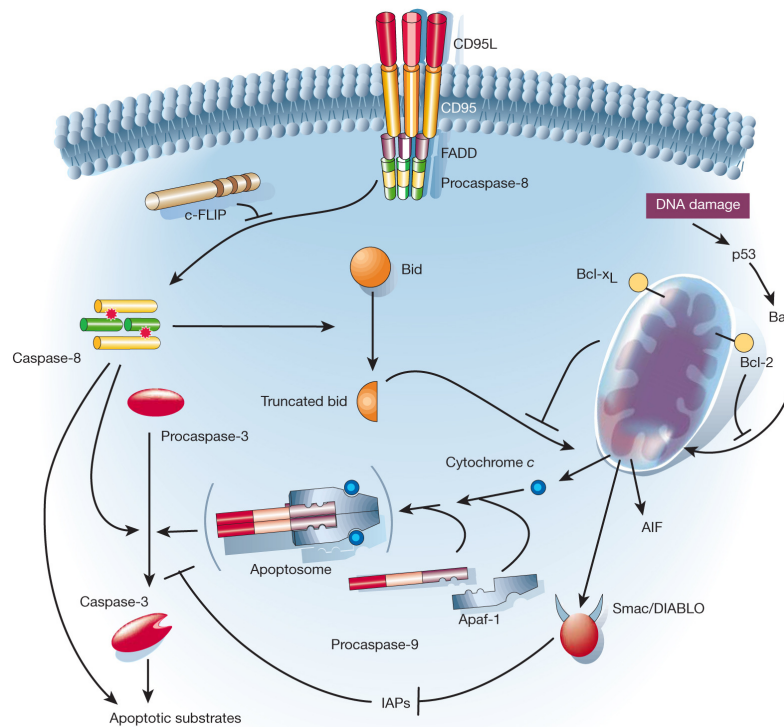


Fig. 8: Schematic view of the extrinsic and the intrinsic apoptosis pathway ^[50]

1.2.3. Mitochondrial pathway (intrinsic pathway)

Due to apoptotic signals pro-apoptotic proteins belonging to the Bcl-2 family experience modifications which activate and translocate them to mitochondria to induce the intrinsic apoptotic pathway (Fig. 8).^[48] The consequence is disruption of the mitochondrial membrane and loss of the electrochemical potential following a release of cytochrome c, Smac/DIABLO and apoptosis-inducing factor (AIF). Cytochrome c forms the apoptosome by interacting with Apaf-1 (apoptotic protease activating factor-1), ATP and pro-caspase-9 in the cytosol. Subsequent activation of caspase-9 leads to the initiation of the caspase cascade.^[43]

The type of signals cells receive determines which pathway is switched on. Extracellular signals like growth factors, hormones, cytokines and toxins elicit the extrinsic pathway, whereas hypoxia, oxidative stress, heat shock, DNA damage, viral infections and radiation trigger the intrinsic pathway. However, a cross-talk between both pathways exists.^[51] In some cell types caspase-8 is insufficient to induce the receptor-mediated pathway. Thus interaction with the mitochondrial pathway is a way out of the misery. Activated caspase-8 cleaves the pro-apoptotic Bcl-2 member Bid, which travels to mitochondria and induces cytochrome c release.^[39]

Both pathways join the same final route to cell death. Caspase-8 or -9 in the case of the mitochondrial pathway activate pro-caspase-3, an effector caspase, which in turn activates caspase-6 and caspase-7. Cellular targets are proteins like nuclear lamins, the cytoskeletal protein actin, DNA repair proteins like PARP-1, Bcl-2 family members, transcription factors (e.g. NF- κ B) and cell cycle regulators (e.g. pRb). The final result is organized cellular death.^[52]

1.2.4. Regulation of apoptosis

Referring to the regulation of apoptosis the same as for cell cycle regulation is valid. Proper regulation is a strict necessity, whereas control is executed at the level of caspases, involving the FLIP and Bcl-2 family, as well as inhibitors of apoptosis (IAPs) as important regulatory proteins.^[40] Although there are many other regulatory levels, not all will be mentioned here.

1.2.4.1. FADD-like ICE inhibitory proteins (FLIPs)

FLIP proteins are involved in the receptor-mediated pathway and bind to the FADD adaptor protein thus blocking the caspase binding site. Alternatively they are also able to bind to pro-caspase-8/10 directly to inhibit their own binding to FADD. DISC formation and apoptosis are impeded. ^[52]

1.2.4.2. Bcl-2 family

The Bcl-2 family includes anti-apoptotic (e.g. Bcl-2, Mcl-1, Bcl-X_L) as well as pro-apoptotic (e.g. Bax, Bad, Bim) factors which are important in the control of the mitochondrial membrane permeability. ^[48] They decide whether apoptosome formation takes place or not, whereas an imbalance of one of both protein members is decisive. ^[50]

Anti-apoptotic proteins reside usually in the outer mitochondrial membrane of healthy cells ^[53] inhibiting the conformational change of pro-apoptotic proteins, thus avoiding mitochondrial breakdown. When pro-apoptotic proteins, activated by death signals, undergo a conformational change they travel to the surface of mitochondria and bind to their Bcl-2 counterparts. The result is inactivation of anti-apoptotic proteins and pore formation. ^[39] In spite of everything the concrete Bcl mechanism is still unclear. ^[54]

Bad is a typical pro-apoptotic protein kept inactive in healthy cells by phosphorylation. Its sequestration in the cytosol is mediated by binding to 14-3-3 protein. Upon apoptotic stimulation dephosphorylation and translocation of Bad to the mitochondria induces apoptosis. ^[39]

1.2.4.3. Inhibitors of apoptosis (IAPs)

Inhibitors of apoptosis (e.g. survivin, XIAP, c-IAP1/2) are proteins affecting both apoptotic pathways. ^[52] They directly interfere with caspase-3, -7 and -9 and inhibit their proteolytic procession required for full caspase activity. Admittedly it was not reported that other caspases are affected. ^[55]

Smac/DIABLO (second mitochondria-derived activator of caspases), a protein released together with cytochrome c from mitochondria during apoptosis, is a typical IAP antagonist. Apparently it blocks the IAPs thereby promoting caspase cascade and apoptosis. ^[39] Due to the fact that IAP proteins contain a RING finger domain, typical for E3 ligases, and it is conceivable that they might target themselves or caspases for proteosomal degradation. ^[56]

Survivin is an essential inhibitor of apoptosis with cell cycle regulatory functions during mitosis at the same time. In contrast to other IAPs survivin has an unusual structure containing only one typical BIR domain. ^[57] During the spindle checkpoint in mitosis phase survivin is phosphorylated at threonine³⁴ by CDK1-cyclinB complexes. Thus active survivin controls the formation of the mitotic spindle by binding to microtubules and centrosomes and prevents apoptosis at the same time. ^[58] Concerning its apoptotic functions it affects indirectly caspases, it is able to bind to XIAP and to prevent its Smac inhibition (thus promoting mitosis at the same time) and it can block apoptosome formation thus promoting survival. ^[59]

1.3. Necrosis

Necrosis is in contrast to apoptosis a form of uncontrolled cell death induced by accident. Typical characteristics are cellular swelling and loss of membrane integrity with a concomitant release of cytoplasmic content. Thereby adjacent cells are affected and inflammation occurs eventually. Usually treatment of cells with high cytotoxic agents provokes necrosis. In literature necrosis is often termed as oncosis. ^[41]

1.4. Cancer

Cancer or malignant neoplasm as it is called medically is a genetic disease which can be traced back since mankind exists. ^[60] In consequence of deregulation of cell cycle cancer cells display a common uncontrolled proliferation and can be distinguished from healthy cells by several traits. ^[61]

1.4.1. Characteristics of cancer

In contrast to normal, healthy cells tumor cells acquire particular properties summarized in Figure 9, thus making them different. According to Hanahan and Weinberg one of the six characteristic features of cancer cells is **escape from dependency on growth factors**. On the one hand cancer cells are independent of mitogenic signals generating their own stimuli to overcome cell cycle repression. On the other hand malignant cells are deaf to inhibitory signals. A farther cancerous property is **evasion from apoptosis** by affecting the tumor suppressor protein p53, thus promoting survival. The **unlimited replicative potential** of tumor cells comes from their ability to activate telomerase to maintain telomere length and to prevent that way cellular aging. In order to expand, cancer cells induce **angiogenesis**

(blood vessel growth into the tumor), an ingenious trick which provides them enough oxygen and nutrients. At this stage cells are finally capable of **invading tissues and** spreading all over the body **forming metastasis**, a crucial step to consecrated downfall. ^[62]

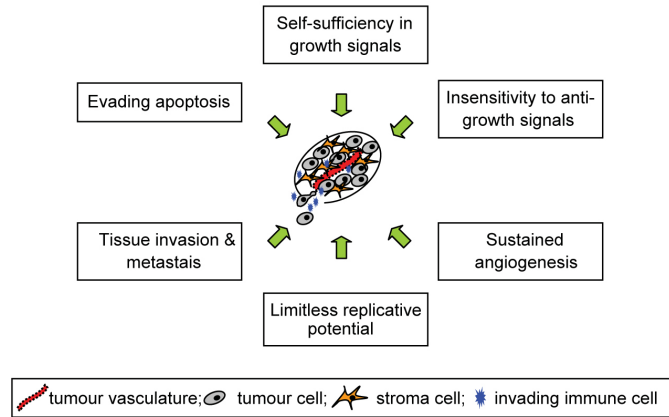


Fig. 9: Properties of cancer cells ^[63]

1.4.2. Malignant transformation leading to cancer

Malignant transformation refers to the stage-wise development from healthy to tumorigenic cells towards cancer. Independent of the cancer type a common sequence of events can thereby be observed.

Cancer results from spontaneous (e.g. replication errors) or environmental induced (e.g. UV radiation, chemicals) genomic mutations in important regulatory genes. These mutations include point mutations, frame-shift mutations, chromosomal mutations as well as epigenetic modifications (and many more). ^[64, 65] However, a simple mutation per se is only harmful when propagated and anchored in the progeny's genome. Nevertheless, this does not immediately lead to the development of cancer, but it is a step forward to it. Clonal expansion of cells, which harbour a mutated gene, increases the chance for another mutation to occur. ^[65] After accumulation of several mutations in different genes – until now it is not known how many of them are required to induce malignant transformation – DNA damage, altered gene expression and protein function cause genomic instability. ^[65] Thus, according to different reports many cancer types display chromosomal aneuploidies. ^[64, 66] Although cells have certain DNA repair mechanisms, they seem to fail in most of the cases over the length of time. ^[65] Anyway, besides somatic mutations hereditary transmitted germline mutations represent a risk factor for cancer development as well. ^[64] Altogether cancer obviously seems to be a complicated multi-step process of malignant cell transformation during a long time span.

Two major gene classes seem to contribute to malignant transformation to a very great part: proto-oncogenes and tumor suppressor genes.^[67] Alterations in these genes lead to development of cancer.^[68]

1.4.2.1. Proto-oncogenes

Proto-oncogenes are genes encoding proteins important in cellular stimulation regarding promotion of cell division and inhibition of apoptosis.^[65] In spite of a tight gene regulation mutations in proto-oncogenes resulting in a “gain-of-function” cause cellular transformation. Such mutations often imply protein overexpression, constitutive activation or failure in switching off protein expression.^[30] Due to a dominant nature, a mutation in a single allele is enough to enhance oncogene stimulatory properties.^[65] Aberrant activated proto-oncogenes are then called oncogenes.^[30]

Different transcription factors, chromatin remodelers, growth factors and their receptors, signal transducers (e.g. *Ras*) and apoptosis regulators (e.g. Bcl-2 family) are counted among oncogenes.^[68]

1.4.2.1.1. *Ras* oncogene

One of the most famous oncogenes is the *Ras* oncogene. *Ras* is a small GTP binding molecule residing in the inner plasma membrane. It is involved in transducing a mitogenic signal from a receptor on cell surface to important downstream kinases, thus mainly activating the Raf-MEK1/2-ERK1/2 MAP kinase pathway. This activation is a prerequisite for cells to enter G₁ phase of the cell cycle. Other *Ras* targets are Ral-GEFs and PI3-K (phosphatidylinositol-3-kinase) which are important in remodelling the cytoskeleton.^[69]

Ras normally is inactive when bound to GDP (guanosine diphosphate). Due to stimulatory signals recognized by cell surface receptors the activated GEF (guanine exchange factor) catalyzes the exchange of GDP to GTP (guanosine triphosphate), which is present in a higher concentration in the cytoplasm. GTP-bound *Ras* constitutes its active form and *Ras* can exert its functions. However, *Ras* activation is short-termed. GAPs (GTPase activating protein) stimulate the intrinsic hydrolyzing potential of *Ras*: upon hydrolysis GTP is converted to GDP and *Ras* gets again inactivated.^[69]

In many tumor types (about 70 %) *Ras* is mutated. This leads to its overexpression and/or a constitutive activation and in a consequence to enhanced signal transduction and acceleration of the cell cycle progression. Thus *Ras* contributes to neoplasm formation.^[69]

1.4.2.2. Tumor suppressor genes

Tumor suppressor genes are the adversaries of proto-oncogenes. They have inhibitory effects on the cell cycle, thus impeding progression if necessary. ^[65] Mutations in tumor suppressor genes result in their inactivation. Thus important control mechanisms fall away and proliferation can unhampered proceed. ^[30] Unlike in oncogenes, mutations of suppressor genes are recessive, this means that a mutation in one allele is not enough to disable the affected gene. Nonetheless, it represents a predisposition towards tumor formation. Once a mutation in the second allele is acquired the gene is completely inactivated. This observation has become famous as the two-hit hypothesis proposed for the tumor suppressor pRb by Knudson A. in 2001, but is valid for all tumor suppressors. ^[70, 71, 72] Tumor suppressors are usually proteins which are important in controlling checkpoints or programmed cell death. ^[65]

1.4.2.2.1. Retinoblastoma protein (pRb)

The retinoblastoma protein is an important tumor suppressor protein that regulates the transition of the G₁ restriction checkpoint during cell cycle. ^[73] As depicted earlier pRb is a prosperous inhibitor of transcription during S phase of the cell cycle. Hence, its correct inactivation is required for cellular proliferation. ^[74]

Rb contributes to malignant transformation, if changes affecting the corresponding gene or its encoded protein occur. Inactivation of the *Rb* gene due to mutations (DNA-replication or mitotic recombination errors) ^[73] results in an allelic defect, which can be transmitted through the germline. ^[64] Later during child development a second acquired somatic mutation in the other allele evolves to retinoblastoma. Retinoblastoma is a retina neoplasm in the eye. ^[73] At a very low probability retinoblastoma can also develop due to two separated somatic mutation events in both alleles. ^[30]

In contrast to mutations which prevent *Rb* gene expression, alterations regarding the protein of Rb (phosphorylation, degradation, viral protein interaction) ^[74] lead to a non-functional protein unable to bind E2F. The consequence is an uncontrolled proliferation of cells contributing to the development of other cancer types than retinoblastoma. ^[17]

1.4.2.2.2. Transcription factor p53

The transcription factor *p53* (*TP53*) is one of the most important tumor suppressor genes which got famous by its second name “the guardian of the genome”. ^[75, 30] Due to its regu-

latory functions in cell growth and genomic stability p53 protects cells from malignant transformation. ^[76, 77]

Under normal conditions p53 is present only at low concentrations due to limited stability. It has a short half life (about 20 minutes). ^[76, 77] In response to DNA damage, hypoxia (oxygen deficiency) or other cellular stress stimuli ^[78] p53 translocates to the nucleus where different kinases (e. g. ATM, ATR, DNA kinase), which are induced dependent on the kind of stimuli, phosphorylate it on different serine or acetylate it on lysine residues (different other modifications can occur as well). ^[76, 77] These modifications stabilize and thus activate p53 subsequently. Its concentration rises and formation of p53 tetramers (consisting of two p53 dimers) takes place. p53 is then able to bind to DNA and act as a transcription factor. ^[30] Downstream targets include the CDK inhibitor p21^{Cip1/Waf1}, MDM2, GADD45, cyclin G, Bax and IGF-BP3. ^[77]

There are two different outcomes of p53 activation. p53 either induces cell cycle arrest or programmed cell death. Which process is finally triggered, depends on multiple factors. Growth and survival stimuli, pRb overexpression, presence of p21^{Cip1/Waf1} and cellular concentration of p53 itself seem thereby to play an important role in this decision. ^[79]

p53 induced upregulation of p21^{Cip1/Waf1} protein early in cell cycle ^[30] leads to the inhibition of cyclin D-CDK4/6 complexes and prevents a further progression through the cell cycle. Arrest of cells in G₁ phase is the result. ^[79] In G₂ phase p53 cannot induce cell cycle arrest, but plays a role in maintaining it. Upon DNA damage p53 targets several downstream proteins. p53 negatively affects the cdc25C phosphatase which is important for activation of the mitosis promoting factor (MPF), CDK1-cyclinB. ^[80] Additional upregulation of 14-3-3-σ prevents the correct nuclear localization of MPF thus impeding cell cycle progression. ^[81] p53 induced p21^{Cip1/Waf1} expression seems to affect CDK1-cyclinB by inhibiting its kinase activation by CAK ^[82] or by sequestering the complex in the nucleus to prevent the activating dephosphorylation by cdc25C phosphatase. ^[83] p53 also activates the GADD45 protein (growth and DNA damage protein), which normally interacts with PCNA, but which also seems to interact with CDK1 leading to its dissociation from cyclin B. ^[84]

Cell cycle arrests comprise a perfect chance for cells to switch on various DNA repair mechanisms to remediate the induced errors. If the repair was successful, cells can re-enter the cell cycle to grow farther. Otherwise apoptosis would be the logical consequence. ^[85]

Regarding p53 induced apoptosis, p53 binds to promoters of various pro-apoptotic proteins and activate their transcription. Such proteins include Bcl-2 family members (PUMA,

NOXA, Bax), p53^{AIP1} (p53 apoptosis inducing protein), Apaf-1 and Fas. Thus p53 is able to influence the extrinsic and intrinsic pathway.^[76, 77] However, a transcription independent mode of p53 action was described as well. p53 is believed to translocate into mitochondria^[86], where it contributes to the loss of membrane integrity leading to caspase activation and thus to apoptosis.^[87] Other theories say that p53 directly interacts with the pro- and anti-apoptotic members of the Bcl-2 family.^[76, 77]

p53 itself is regulated by Mdm2 (mouse double minute 2) protein, an E3 ligase which binds to the transactivation domain of p53 and blocks its function. On top of it Mdm2 targets p53 for polyubiquitination and proteosomal degradation. Mdm2 is a downstream target of p53 at the same time and thus transcribed by the latter. This negative feedback loop assures that p53 levels settle down after a triggered response.^[79] The CDK inhibitor p14^{ARF} (alternative reading frame protein) in contrast is able to bind to Mdm2 and to prevent p53 degradation. Additionally, all p53 modifications induced upon genotoxic stress contribute to a disruption of the interaction with Mdm2.^[88]

In 50 % of all tumors *TP53* is mutated, in all other cases, proteins influencing p53 are affected.^[89] In particular the DNA binding domain of p53 seems to be very susceptible for such mutations, an evidence for its importance in the p53 suppressor functions.^[90] Additionally, *TP53* germ line mutations occur. They got well known as the Li-Fraumeni syndrome in which affected persons develop a tumor at a juvenile age and have a great chance to develop secondary carcinomas as well. Apart from occurring mutations p53 can also be inactivated by virally encoded proteins.^[91] A more detailed description about these mechanisms can be found in the next section.

Hence, p53 is definitely the focus of tumor development and there is a great interest in restoring p53 functions by p53-based therapies, like for instance gene therapy or reconstitution of p53 wild type conformation.^[91]

1.4.3. Other targets of cancer

In addition to oncogenes and tumor suppressor genes, which are affected most of the time in neoplasia, several other deregulations may contribute to malignant transformation. They should be mentioned here shortly. In association with the cell cycle overexpression of cyclins, aberrant activation of CDKs^[92], defective checkpoints and mutated CDK inhibitors abrogate the correct cell cycle progression.^[10] Overexpression of anti-apoptotic proteins or IAPs like survivin, mutations in death receptors^[39], failure in DNA repair and virally transformed cells are also often observed.^[8] Anyway, nearly every protein equipped with

regulatory functions seems to be a potent target in cancer. Unfortunately not all of them are identified yet, but with the information available anti-cancer treatment are on the advance. Last but not least, it should be mentioned that not only internal factors play important roles in cancer development. Cancer has a lot to do with lifestyle and attitude, this regards especially dietary, alcohol consumption and smoking, three main risk factors for developing cancer.^[93]

1.5. Human papillomaviruses (HPV)

Human papillomaviruses are small, non-enveloped viruses containing a double-stranded, circular DNA of 8 kb length.^[94] Papillomaviridae display a high affinity for epithelia, infecting dermal or mucosal tissue of the anogenital and oropharyngeal tract.^[95] Viruses infecting the mucosa can be classified in low-risk and high-risk subtypes.^[94] Low-risk viruses, including HPV **6**, **11**, 42-44, 53-55 and 66^[96] cause oral papillomas, skin warts or condylomata in the anogenital area and are considered to be inoffensive.^[97, 98] In contrast, high-risk viruses are able to transform host cells^[94], long-term infections thus provoking cancer of the vulva, vagina, cervix, anus and penis.^[97] HPV **16**, **18**, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82 and 83 are considered as being intermediate or high-risk subtypes.^[99] Bold highlighted numbers represent the most frequent HPV types detected.^[96] Up to now 200 virus types have been discovered^[94], whereas HPV occurs in 20 different species for instance in birds.^[100]

1.5.1. HPV genome structure

HPV particles encode eight open reading frames (ORFs), which are translated from a polycistronic mRNA.^[94] Thereby three different types of genes can be distinguished (Fig. 10). The late genes L1 (major capsid protein) and L2 (minor capsid protein) assemble the capsid, a protein coat around the DNA to protect it.^[101] The early genes E1, E2, E4 and E5 are essential for DNA transcription and replication, whereas the E6 and E7 proteins are important for cellular transformation.^[98] And ultimately a non-coding region between the early and the late genes, a stretch of 1 kb which is called the upstream regulatory region (URR) due to its regulatory functions in gene expression, replication and viral assembly.^[101]

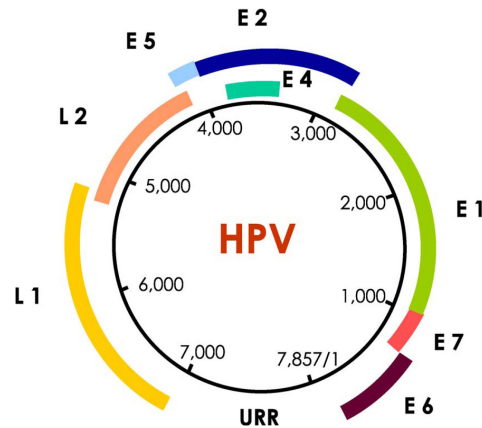


Fig. 10: General genome structure of human papilloma viruses – E1, E2, E4-E7 = early transcribed genes, L1 and L2 = late transcribed genes ^[101]

1.5.2. HPV life cycle

The life cycle of human papilloma viruses strongly depends on their host, the keratinocytes. ^[94] This is due to the fact that HPV does not encode his own replication proteins and therefore parasitically exploits the DNA replication machinery of its host. That means viruses replicate only when the host is copying its DNA. ^[102]

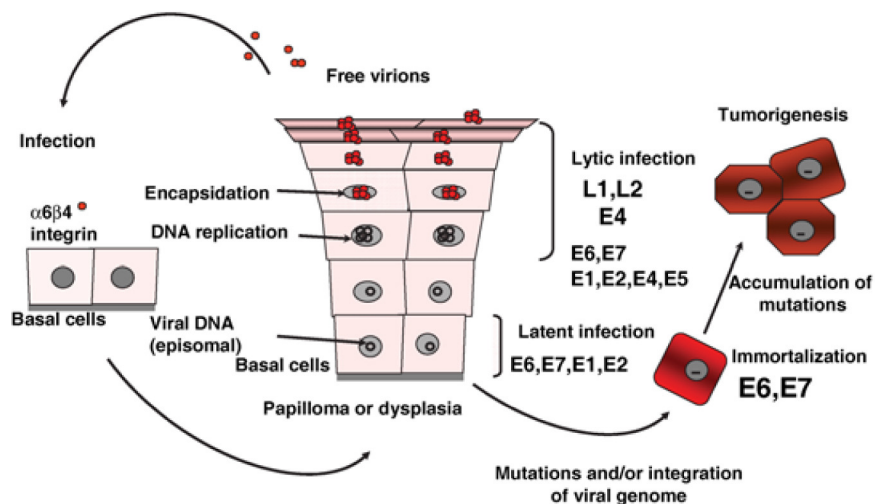


Fig. 11: HPV infection pathway of keratinocytes ^[103]

Figure 11 depicts the HPV infection pathway. Human papilloma viruses infect basal stem cells of the epithelium, reaching them by small skin injuries. ^[101] These cells are the only type of cells they can enter ^[102], because of their continuous dividing properties. All other epithelial cells, lying over the basal cells, are differentiated and not able to divide anymore. Hence, they are useless for viruses. ^[101]

Access to cellular insides is achieved by the binding of viral particles to two different cell surface receptors, which enable their uptake. However, until now it is doubtful which receptors exactly are in charge. Heparan sulfate proteoglycan and $\alpha 6$ integrin receptors are thought to be involved.^[104] Entry of virions is finally mediated by endocytosis of clathrin coated vesicles, which seems to be delayed however.^[105] Viral uncoating, a scientific term for the release of viral DNA from the capsid, occurs in late endosomes and/or lysosomes. The DNA is then imported to the nucleus, where replication can take place.^[104] The L2 protein seems to be involved thereby since it contains a nuclear localizing signal (NLS).^[94] In basal, undifferentiated cells the viral DNA remains inside the nucleus episomal, that means it does not integrate into the genomic DNA of the host.^[98] The first genes to be transcribed are E1 and E2^[94, 106], both involved in viral replication and episomal maintenance. Initiation of early gene transcription is mediated by cellular transcription factors upon binding to the URR.^[106, 100] E1 and E2 are expressed at low levels and produce only a low copy number of the viral DNA. E6 and E7 expression in basal stem cells seems to be in question yet.^[100] As being a helicase, E1 has the ability to unwind the DNA and additionally to interact with DNA polymerase and different accessory proteins to induce replication. However, its DNA binding is of low affinity. Therefore E2, a DNA-binding protein, helps in recruiting E1 to the origin of replication.^[94, 100, 104] Besides, E2 displays transcriptional regulatory functions, thus controlling E6 and E7 expression from the early promoter.^[104]

After mitosis of basal stem cells one of the daughter cells normally migrates upwards through the various skin layers and starts its differentiation program. This is linked to the final exit of cells from the cell cycle and marks the end of their dividing properties.^[94, 103] At the top cells lose their nuclei^[94], get a cornified stratum and replace the old cells, which flaked away. This is part of the skin regeneration process in healthy cells.^[102] In infected cells HPV senses the induced differentiation and renders suddenly its transcription program to enhance gene expression. E4, E5, E6 and E7 proteins are now transcribed at high levels. Before, hardly any of these proteins were synthesized.^[106] Upregulation of E6 and E7 proteins leads to a continuous cell proliferation and terminates the differentiation program of cells. Cells thus replicate at an accelerated rate. This may cause the formation of benign warts. In the worst case however, cells are being transformed by E6 and E7 activities and as a result exposed to a higher risk to develop cancer. About E4 and E5 proteins very little is known. E5 is said to be involved in mimicking growth signals to keep cells proliferating^[102], whereas E4 helps in disrupting the keratin network in cornified cells at

upper cell layers.^[104] Eventually expression from the late promoter leads to L1 and L2 accumulation in cells of the external epidermal layer. This stimulates packaging of new progeny viruses, which are then shed together with old skin cells. New viruses escape from the cornified stratum assisted by E4.^[104]

Human papillomaviruses evade from immune system by their perfect elaborated strategy to survive in keratinocytes. Replication and virus particle packaging is restricted to cells in the upper layers of the skin/mucus, far away from any immune competent cells.^[102] Likewise, HPV oncoproteins can interfere with interferons and reduce the MHC I expression in infected cells, thus they are not able to present the antigen to the immune system.^[107] So the infection may not be observed for a long time.^[102]

1.5.3. HPV E6 and E7 oncoproteins

The E6 and E7 proteins are both mainly nuclear proteins^[108], expressed relative early during the viral life cycle.^[104] Their ability to promote continuous cell proliferation by the abolishment of the proper cell cycle regulation is of great significance in establishing viral pathogenesis.^[108] However, only cells infected by high-risk papillomaviruses seem to be at risk to develop cancer.^[104] Although E6 and E7 from low-risk HPV types drive cells into an accelerated progression as well, the result is simply an accumulation of cells forming a benign wart, which is restricted to a small area.^[98, 102] These oncoproteins become only dangerous when the HPV infection persists over years. Then the probability of random integration of the viral DNA into the host's genome increases. It is an event which does not provide any benefit for the virus, it is rather contrariwise because most of the genes are lost thereby. The infection can usually be cleared, but some of the cells may survive with the integrated E6 and E7 oncoproteins in their genome. This would be a devastating consequence, which allows mutations to accumulate.^[102]

E6 and E7 oncoproteins are expressed from a bicistronic mRNA^[109] and seem to cooperate together.^[104]

1.5.3.1. E6 oncoprotein

A step forward to cellular transformation is made by the different interactions of the E6 oncoprotein with several binding partners inside the cell. These interactions result in the inhibition of the proteins.^[98]

The probably most important binding partner of E6 is the tumor suppressor p53, which is normally expressed and maintained at low levels in healthy cells. As the viral infection occurs, the cell reacts with the upregulation of p53. To impede cell cycle arrest or apop-

tosis induced by p53, E6 grabs p53 and binds additionally to E6-AP (E6 associated protein), an E3 ubiquitin ligase, at the same time. This allows the interaction between p53 and E6-AP, which normally would not be able to bind to p53 on its own. As a matter of fact p53 is ubiquitinated and degraded in the proteasom (Fig. 12).

However, E6 can inactivate the p53 protein by other mechanisms discovered recently. The interaction with p53 occurs in a way that blocks its DNA binding domain thus preventing its transcriptional functions. Secondly, E6 can bind and mask the NLS of p53, thereby sequestering it in the cytoplasm keeping it away from its target action side. A third mechanism is the binding of E6 to CBP/p300, proteins which normally activate p53. ^[108] E6 also targets proteins important for apoptosis like FADD, Caspase-8 and Bak and mediate their degradation in order to reduce the probability of an induced apoptosis due to the viral infection. Many other interaction partners involved in chromosomal stability, epithelial organization, differentiation and transcriptional regulation have been reported as well. ^[98]

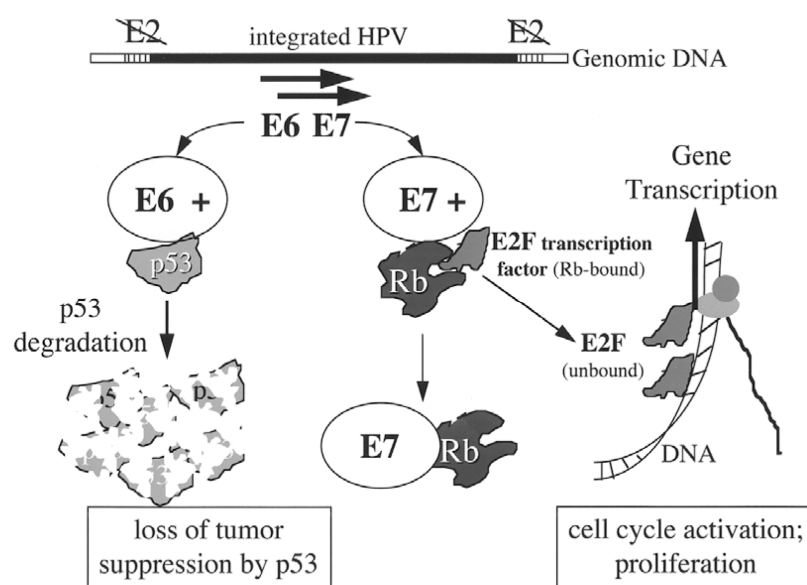


Fig. 12: E6 and E7 oncogene interaction with important cellular tumor suppressor proteins ^[110]

1.5.3.2. E7 oncoprotein

The high-risk E7 oncoprotein leads to immortalization of cells by interacting with the tumor suppressor pRb and its binding partner E2F, outlined in Figure 12. pRb normally controls the G₁ restriction checkpoint through inactivating the S phase specific E2F transcription factor. Binding of E7 to hypophosphorylated pRb and concomitantly to E2F displays the latter from the complex. ^[111, 112] Subsequently E2F induces transcription of genes important for DNA replication. ^[101] Thus, E7 abrogates the restriction checkpoint and medi-

ates cell cycle progression. However, E7 complements this by the interaction with different cellular proteins. Interaction with CDK inhibitors (p21^{Cip1/Waf1}, p27) abolishes their inhibitory function of halting the cell cycle. E7 binding to HDACs (histone deacetylases) allows chromatin remodelling processes to take place and to induce gene transcription. Additionally, binding to CDKs, p300/CBP, TBP and AP-1 transcription factor were observed as well. ^[111, 112]

Anyway, unscheduled S phase activates apoptosis, triggered by p53. At this stage E6 intervenes and interacts with p53 directly to prevent undesired cellular changes. ^[113]

1.5.4. Cervical cancer

Cervical cancer is the abnormal growth of cells in the lower part of the uterus, the so-called cervix (neck of uterus). ^[103] This type of cancer causes very frequently female death worldwide. ^[114]

Human papillomaviruses have been accounted for more than 90 % of all cervical cancers ^[111], whereas in particular HPV types 16 and 18 seem to be responsible for 70 % of all cases. However, an HPV infection is not sufficient to induce cancer. Several other factors like taking hormonal contraceptives over a longer period of time, smoking, various other coinfections (e.g. chlamydia, herpes simplex virus) and bad nutrition contribute to tumor formation. ^[101]

1.5.4.1. Transmission and diagnosis

Human papillomaviruses are transmitted by skin-to-skin contact, which occurs to a great part by sexual intercourse. Thus, frequent changing of multiple sexual partners increases the risk of infection. Thereby using condoms seems not to protect entirely because of the possible contact to other infected parts of the anogenital tract. ^[102, 115] Since HPVs are quite resistant to environmental impact they are able to survive and to establish a source of infection. ^[115] Vertical transmission from mother to child through the birth canal is also at question. However both occur to a lower extent. ^[116]

A majority of the population is infected with various types of HPV without even knowing about it. Thus the danger of infection is quite high. One possibility to prevent or at least to reduce the outbreak of cancer in most cases is early diagnosis.

The first method to be used for HPV screening was the Papanicolaou test or shortly Pap smear. It is a simple staining method of cells scraped off from the cervix. Examination under the microscope allows the differentiation between healthy and abnormal cells, which

display enlarged nuclei surrounded by clear rings (Fig. 13). But the visual inspection of the cervix is part of the daily routine as well. ^[102, 115] Since detection of HPV by Pap smear is used, 70 % of cancer mortality could be reduced in the past 50 years in the United States. ^[102] However, due to false negative results, which can occur due to clumping of cells, other detection methods are additionally performed. ^[115]

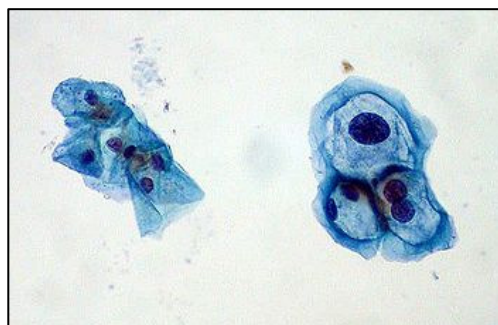


Fig. 13: Pap smear of cervical cells – Cells on the left are healthy; cells on the right display enlarged nuclei, which indicate an HPV infection. ^[117]

A better and more sensitive detection method is DNA testing, where cervical biopsies are taken to extract cellular DNA or RNA. Exposure of the genetic material to special reagents generates a positive signal, if HPV DNA/RNA is present. Two methods are generally in use: in-situ hybridization and PCR-hybridization.

During in-situ hybridization the isolated genomic material is exposed to probes which bind (hybridize) to specific HPV sequences, if they are present in the sample. Incubation with an antibody recognizing the probe itself enables the visualization of a positive signal, if a detection reagent is added. PCR-hybridization is a similar technique, which in contrast amplifies the extracted genetic material before beginning the hybridization. For this reason it is more sensitive than hybridization alone.

The advantage of these sensitive methods is that infected cells can be detected even before any cytological abnormalities occur. ^[102]

1.5.4.2. Symptoms and treatment

HPV infections produce seldom any symptoms ^[115, 118], although pain and vaginal bleeding were reported in very rare cases. ^[119] The occurrence of warts can be a typical evidence for an HPV infection. ^[118]

If cytological abnormalities attributed to an HPV infection were detected, appropriate treatment should succeed as soon as possible to impede the further progress. Otherwise the situation would only worsen.

Normally, a healthy immune system is capable of clearing HPV infections in 1-3 years without even to be noticed actually. ^[115] Should the infection persist and erupt visibly, removal of affected cells by surgery, liquid nitrogen, laser, radiotherapy, chemotherapy, chemical burning with salicylic acid or podofilox or by the treatment with antiviral and immunomodulatory agents like interferons takes place. ^[102, 115]

1.5.4.3. HPV vaccines

In addition to the above mentioned treatment types, two prophylactic HPV vaccines are commercially available since 2006. Gardasil[®] from Merck and Cervarix[®] from GlaxoSmithKline are both protein subunit vaccines using the L1 capsid protein of HPV. ^[120, 121] L1 is said to self-assemble into virus-like particles (VLPs) and to elicit an adaptive immune response producing neutralizing antibodies. ^[121]

Gardasil[®] is a quadrivalent vaccine containing L1 proteins from HPV 6, 11, 16 and 18 produced recombinantly in the yeast *Saccharomyces cerevisiae*. This vaccine is believed to protect against 90 % of all genital warts and against 70 % of cervical cancer. Cervarix[®] in contrast is only a bivalent vaccine protecting against HPV 16 and 18 and is produced by baculoviral expression in the insect cells *Trichoplusia ni*. ^[107, 120] However, both vaccines do not protect against persistent HPV infections acquired before vaccination. ^[120] Therefore the best time for immunization is during youth, when young women have not become sexually active and are not infected yet. Hence, vaccination between 9 and 26 years is recommended. ^[122, 123, 124] Currently no one knows if refreshment to boost the immune reaction will be necessary after some time. ^[107, 118]

It is claimed that these vaccines are not dangerous to healthy people. That means that even though viral material is directly injected, they are not able to initiate an HPV infection themselves due to the lack of a viral DNA. Although adverse local reactions after vaccination were quite common. The majority experienced pain at the injection side, swelling and very rare fever. Surprisingly Gardasil[®] can be used to vaccinate male persons to prevent spreading of HPV as well, though it is not admitted everywhere. ^[125]

However, due to the yet unknown consequences of vaccination and the with it associated high costs young women are often restrained from assuming a risk.

1.6. Pharmacological CDK inhibitors as new therapeutic agents

With all the information acquired so far about cell cycle and its regulation, about programmed cell death, cancer and its development and viral HPV infections all contributing

to tumor progression, it will become obvious that there is more behind cancer prevention and therapy. In addition to anti-cancer drugs developed to directly kill tumor cells, the potential of a new approach involving pharmacological CDK inhibitors was discovered recently. These inhibitors are capable of modulating cells in a way that stops the transforming properties of malignant cells and provide a good alternative therapy to vaccines against HPV.^[10]

Pharmacological CDK inhibitors are synthetic compounds^[43] which act like cellular CDK inhibitors. Due to frequent deregulations of cyclin-dependent kinases (CDKs) or their natural inhibitors during malignant transformation, pharmacological CDK inhibitors, in contrast, could offer possible alternatives for therapy. Up to now more than 50 such compounds including flavopiridol, ROSC and olomoucine are investigated in their efficacy to fight cancer.^[126]

A common feature of all pharmacological and natural CDK inhibitors is the binding capability to the ATP binding site of kinases.^[10, 126] Thus, blocking the activation of cyclin-dependent kinases impedes the further cell cycle progression.

These CDK inhibitors are thought to be highly selective and to have anti-proliferative as well as pro-apoptotic effects on cancer cells with a concomitant low cytotoxicity. Some of them are already in clinical evaluation (e.g. flavopiridol and ROSC).^[10, 14, 43, 126] Recent analyses have even discovered that combined therapies with pharmacological inhibitors may be of great success due to possible synergistic effects and that CDK inhibition can be achieved at greater extents, if cells are synchronized prior to the treatment with pharmacological inhibitors.^[127]

However, some of these new anti-cancer drugs are reported to have anti-viral effects as well. They are involved in blocking the viral replication not by inhibiting viral proteins, but cellular ones. Besides viral transcription can be blocked, whereas the cellular transcription is not affected at all.^[128]

So, pharmacological inhibitors constitute a great alternative to highly genotoxic, chemotherapeutic drugs or dangerous HPV vaccines and are worth of further investigation.

1.7. Aims of the present diploma thesis

Considering the fact that pharmacological CDK inhibitors seem to be very effective agents in the treatment of cancer, the question appeared whether small-molecule compounds such as ROSC and OLO II would be able to affect cancer cells lacking functional G₁/S checkpoint. The main purpose of the thesis was to compare the effects of two closely related

pharmacological CDK inhibitors on human HeLa and HTB-31 cervical cancer cells differing in the status of p53 and G₁/S checkpoint. Although structure of both CDK inhibitors is very similar, their properties slightly differ. Therefore, it was of interest to compare their action and to monitor in detail the changes in the expression and functional status of the most important cellular factors regulating cell cycle progression and apoptosis. Moreover, it was important to prove whether cell cycle status of cancer cells prior to the onset of treatment might have any effect on the efficacy of therapy. Following issues were raised:

1. Assessment of the anti-proliferative effects of the two closely related tri-substituted purines roscovitine (ROSC) and olomoucine II (OLO II) on human HeLa and HTB-31 cervix carcinoma cells
2. Determination of pro-apoptotic effects of the two closely related tri-substituted purines roscovitine (ROSC) and olomoucine II (OLO II) on human HeLa and HTB-31 cervix carcinoma cells
3. Examination of the impact of the p53 status on the outcome of treatment of HeLa cells (dysfunctional wt p53 protein) and HTB-31 cells (mutated p53)
4. Determination of the importance of the functional status of cancer cells prior to the onset of treatment for the outcome of therapy
 - a. Effect of CDK inhibitors on asynchronously growing cancer cells after short (10h) and long (22h) recovery period after plating
 - b. Effect of CDK inhibitors on G₁-arrested cervix cancer cells released from TSA block
5. Characterization of the changes in the levels and functional status of major regulators of cell cycle and apoptosis induced after administration of CDK inhibitors

2. MATERIALS & METHODS

2.1. Materials

2.1.1. Cell lines

2.1.1.1. HeLa S₃

Organism	<i>homo sapiens</i>
Organ	cervix
Cancer type	cervix carcinoma
Morphology	epithelial
Medium	RPMI 1640
Fetal bovine serum	10 %
CO ₂ Atmosphere	5 %
Temperature	37°C
Special characteristics	wt p53, HPV-18 positive, lack of G ₁ restriction checkpoint

Tab. 1: Short résumé of HeLa S₃ cell characteristics ^[129]

Human HeLa cells are epithelial cells derived from a primary cervical carcinoma tumor. They are adherent and have the ability to pile up due to loss of contact inhibition. Their population doubling time is approximately 24 hours. Their characteristics are summarized in Table 1, whereas Figure 14 shows an image of asynchronously growing HeLa cells examined under light microscope.

The human HeLa S₃ cervical carcinoma cell line was cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and phenol red as a pH indicator. Passaging of cells occurred twice a week in a ratio varying between 1:6 to 1:10. To maintain physiological conditions as realistic as possible no antibiotics were used for cell culture.

This cancer cell line established from primary tumor is transformed by the high-risk human papillomavirus 18 (HPV 18): multiple copies of HPV 18 DNA are integrated into the host DNA. ^[130] Two of the expressed viral HPV 18 oncoproteins, namely E6 and E7, interfere with important tumor suppressor proteins in HeLa cells. The E6 protein inactivates the p53 protein by enhancing of E6-AP-mediated ubiquitination and degradation in proteasomes. ^[21] The E7 protein binds to the hypophosphorylated pRb, leads to its inactivation and the release of E2F transcription factors, promoting S phase. Thus, the G₁ restriction

checkpoint in HeLa cells is lacking. In general terms, virally encoded proteins abolish cell cycle arrest and induce permanent cell cycle progression.

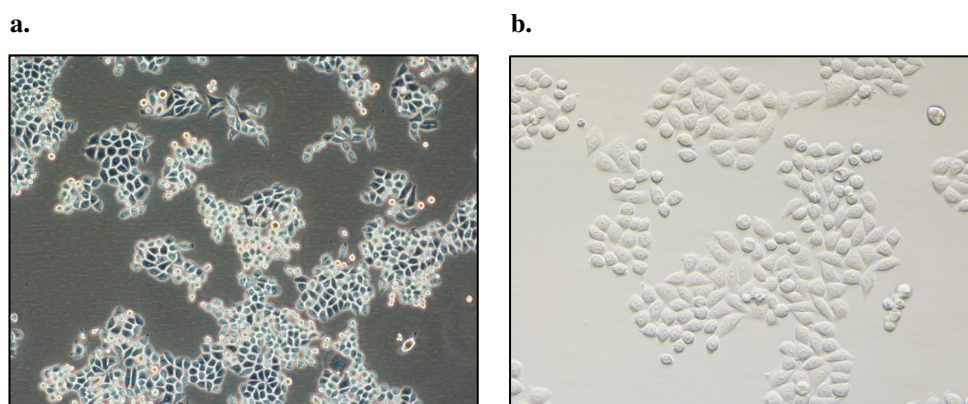


Fig. 14: HeLa cells in cell culture flasks - **a.** a phase contrast image under light microscope, **b.** photography using the Hoffman modulation contrast

2.1.1.2. HTB-31 (C-33A)

Organism	<i>homo sapiens</i>
Organ	cervix
Cancer type	cervix carcinoma
Morphology	epithelial
Medium	MNP
Fetal bovine serum	10 %
CO ₂ Atmosphere	5 %
Temperature	37°C
Special characteristics	mutant p53 and pRb, HPV negative

Tab. 2: Short résumé of HTB-31 cell characteristics ^[131]

Human HTB-31 cells are, like HeLa cells, epithelial cells derived from a primary cervical carcinoma tumor. Table 2 describes their characteristics features, Figure 15 shows their specific morphology.

These cells were cultured in MEME medium supplemented with 10 % fetal bovine serum (FBS), natriumpyruvate, non-essential amino acids and phenol red. Passaging of cells occurred twice a week in a ratio varying between 1:4 to 1:8.

Unlike HeLa cells, HTB-31 cells are negative for any human papillomavirus DNA or RNA, but harbour a p53 “loss-of-function” mutation. A point mutation in its conserved region at codon 273 (Arg → Cys substitution) results in a higher p53 expression. Moreover

retinoblastoma genes are also mutated ($G \rightarrow A$ nucleotide substitution). This results into an in-frame deletion in the pRb protein leading to a truncated form, which is not able to associate with other cellular proteins anymore. As a consequence pRb is hypophosphorylated.^[114] Both mutations contribute to an improper cell cycle progression leading to malignant transformation of cells.

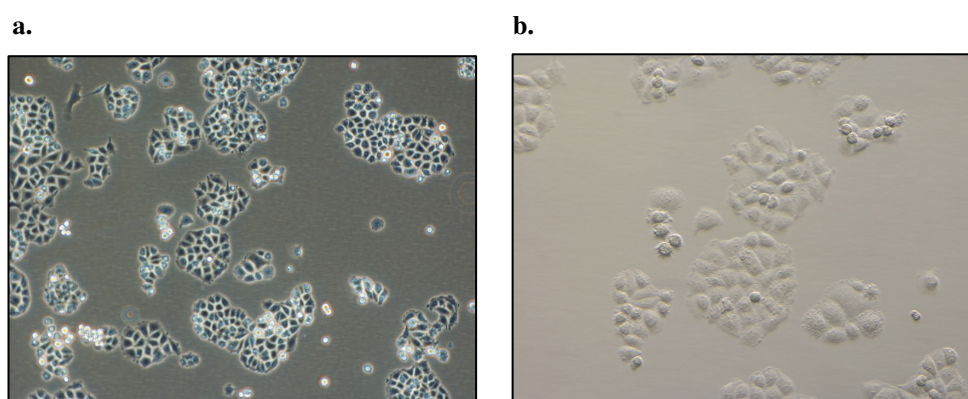


Fig. 15: HTB-31 cells in cell culture flasks - **a.** a phase contrast image under light microscope, **b.** photography using the Hoffman modulation contrast

In general terms, the inactivation of the tumorsuppressor proteins pRb and p53, either by mutation or by inactivation mediated by HPV-encoded oncoproteins, is an important step in human cervical carcinogenesis.^[114]

2.1.2. Drugs

2.1.2.1. Doxorubicin

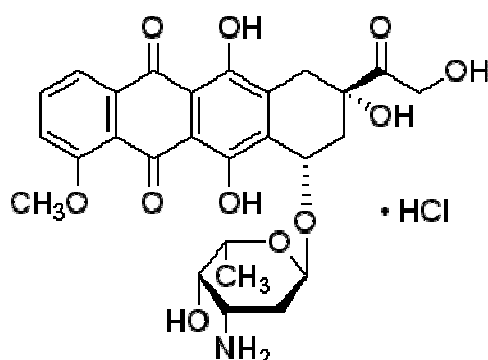


Fig. 16: Molecular structure of doxorubicin hydrochloride^[132]

Doxorubicin (DOX) is an anthracycline antibiotic derived from the bacteria *Streptomyces* var. *caesius*, its molecular structure is depicted in Figure 16.^[133] DOX displays several cytotoxic properties: Doxorubicin DNA intercalation or inhibition of DNA Polymerase

leads to a DNA/RNA synthesis inhibition, interference with DNA unwinding processes, inhibition of topoisomerase II and the generation of free radicals can lead to DNA damage ^[134], but DOX also affects electron transport chains in mitochondrial membranes, thus disrupting them. ^[135] According to the mentioned above action, cellular response to DOX depends on the dosage. At lower DOX concentrations cells undergo a strong G₂ arrest, whereas at higher doses DOX triggers apoptosis. ^[134]

Doxorubicin is highly cardiotoxic and not selective at all, healthy and malignant cells are affected equally. Subsequent secondary effects are severe, thus other more selective and potent anti-cancer drugs are investigated. ^[134]

2.1.2.2. Olomoucine II

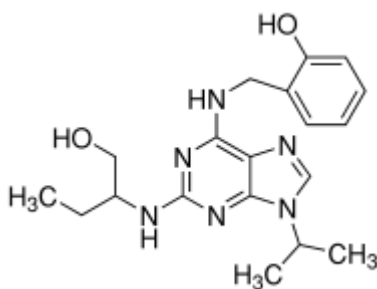


Fig. 17: Molecular structure of OLO II ^[136]

Olomoucine II (OLO II) is a new pharmacological CDK inhibitor that belongs to the purine family, especially inhibiting CDK1 and CDK2. However, it also affects transcription by indirect modulation of the carboxy-terminal domain (CTD) of RNA Polymerase II through CDK7 and CDK9. Although OLO II occupies the active site of CDK2, which is almost the same binding position of ROSC, it seems to be more potent than ROSC itself. Comparing their molecular structures (shown in Fig. 17 and 18) there is a huge similarity, the only difference existing in an additional hydroxyl group at the benzyl ring increasing OLO II's efficiency. OLO II is said to have anti-proliferative effects, blocking the cell cycle and inducing elevated p53 gene expression at higher concentrations. ^[137]

2.1.2.3. Roscovitine

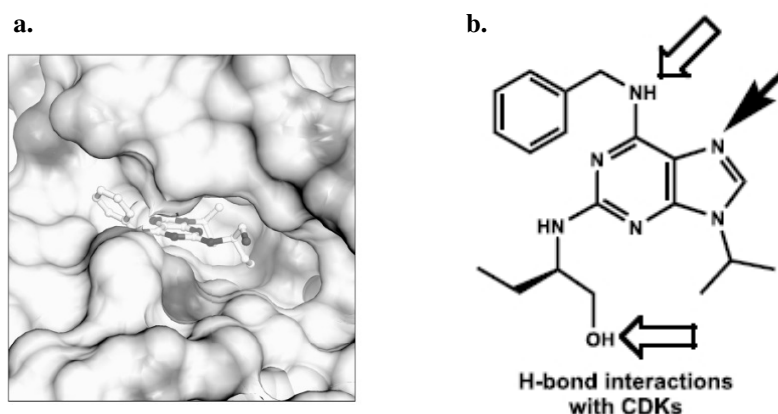


Fig. 18: **a.** Crystal structure of CDK2 in complex with (R)-ROSC, illustrating the position of (R)-ROSC in the ATP-binding pocket, **b.** Atoms involved in H-bond (R)-ROSC-CDK2 binding^[138]

R-ROSC (known also under commercial names Seliciclib or CYC202) is a potent and selective CDK2, CDK1, CDK5 and CDK7 inhibitor.^[138] ROSC, a tri-substituted analogue belongs to the family of purine CDK inhibitors and is, like OLO II, an ATP-like molecule. In other terms, it competes with ATP for CDK binding.^[21] Figure 18 a. shows the crystal structure of (R)-ROSC in the ATP-binding pocket of CDK2. Fig. 18 b. shows the molecular structure of ROSC and the atoms, which are involved in hydrogen bonding to CDKs. In most cell lines two major ROSC effects were observed: a cell cycle arrest and an induction of apoptosis (programmed cell death). Inhibition of the cell cycle progression can occur directly by binding of ROSC to CDKs or indirectly either by interacting with CAK and therefore inhibiting the CDK activating phosphorylation or by stabilizing p27^{Kip1} and enhancing CDK inhibition.^[138]

2.1.2.4. Mimosine

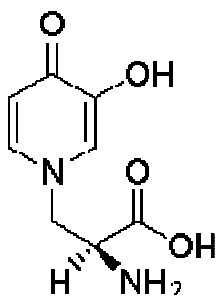


Fig. 19: Molecular structure of mimosine^[139]

Mimosine (MIMO) is a non-proteinogenic, toxic amino acid isolated from the plant *mimosa pudica* (Fig. 19).^[140] Due to its ability to form iron chelate complexes MIMO seems

to repress the synthesis of deoxyribonucleotids and therefore to inhibit DNA replication (mechanism not fully elucidated). Hence, this compound can be used to synchronize cells in late G₁ phase of the cell cycle.^[141] This effect depends on the MIMO concentration and treatment period, but is completely reversible.^[142]

2.1.2.5. Trichostatin A

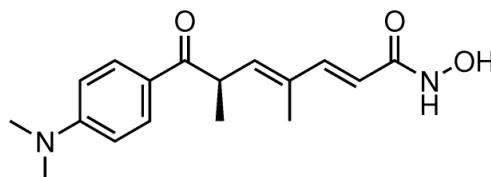


Fig. 20: Molecular structure of trichostatin A^[143]

Trichostatin A (TSA), an antifungal antibiotic, is a mammalian histone deacetylase inhibitor with chromatin loosening effects (molecular structure is shown in Fig. 20).^[144] Chromatin remodelling processes are responsible for transcriptional regulation of genes, whereas histone acetylases (HAT) and histone deacetylases (HDAC) are major key players. Acetylation is usually linked to the activation of gene expression. TSA plays an important role in regulation of acetylation status of histones and therefore also in permanent transcriptional activation of genes. However, acetylation seems to be reversible. Although not all genes respond to TSA treatment.^[145] TSA has also synchronizing effects, arresting cells in G₁ or G₂ phase of the cell cycle, influencing cellular differentiation and causing apoptosis.^[146]

2.1.3. Media

RPMI

400 ml RPMI 1640 (containing L-Glutamine)
10 % FBS
phenol red

Sigma R6504
PAA Laboratories

MNP

400 ml MEME
10 % FBS
non-essential amino acids
natriumpyruvate
phenol red

Sigma
PAA Laboratories
PAA Laboratories

For both media phenol red was used as a pH indicator. Due to a change from a red to a yellow medium colour, overgrown or contaminated cells could be immediately spotted. Storage of media occurred at 4°C in the dark.

2.2. Methods

2.2.1. Cell culture

Material

Cell counting chamber (depth 0.100 mm, 0.0025 mm ²)	Bürker-Türk
Cell culture bottles (T25, T75, T162)	Corning Inc.
FBS (heat inactivated fetal bovine serum)	PAA Laboratories GmbH
CO ₂ Incubator	New Brunswick
Microtiter plates (96-well and 384-well)	Greiner Bio-One
Petri dishes (ø10cm, ø6cm, ø3.5cm)	Corning Inc.
Trypsin/EDTA	Difco Laboratories

For all experiments cells were cultured to 70–80 % confluence in an atmosphere of 5 % CO₂ at 37°C using clear cell culture dishes. It was always a prerequisite to work sterile under a laminar flow in order not to contaminate cells with bacteria or fungi, which would completely influence experimental results. Likewise the medium colour was checked and the cells were examined under the microscope, that refers to cellular morphology, density and adherence status as well as possible contaminations.

For passaging of cells the old medium was removed and cells were incubated for 5 minutes with trypsin/EDTA (limited proteolysis). Thus, detached cells were collected in a sterile falcon tube and centrifuged at 200 x g for 3 minutes at 20°C. Afterwards the old medium was discarded and cells were resuspended in fresh medium. To maintain the cell culture, an appropriate cell amount was transferred to cell culture bottles of different size, containing fresh medium (depending on desired experimental yield:

T25	7 ml medium
T75	15 ml medium
T162	30 ml medium).

To allow a quantitative comparison between different treatments of the same cell line an equal cell number in each experiment was essential. Therefore, the rest of the cell suspension was always diluted to 5 x 10⁴ cells/ml in the case of HeLa cells, whereas HTB-31 cells were plated to 8 x 10⁴ cells/ml (counted in a Bürker-Türk chamber). Differences in cell

density resulted from the fact that HTB-31 cells, also known as C-33A cells, grow slower than HeLa cells (resulted from growth curve analysis, data not shown). So to achieve a reasonably yield of protein amount for subsequent analysis, the cell number was modified. The proper diluted cell suspension was split to cell culture dishes:

ø 10 cm PDs	12 ml of cell suspension
ø 6 cm PDs	4 ml of cell suspension
ø 3.5 cm PDs	2 ml of cell suspension
1 well (96 well plate)	100 µl of cell suspension
1 well (384 well plate)	50 µl of cell suspension
	(2.5×10^4 cells/ml)

Approximately nineteen hours after plating cells were treated with drugs as indicated.

2.2.2. Medications

Material

Sterile filter 0.20 µm (RC15, RC25)	Sartorius
Syringe, 5 ml and 20 ml	BD Discardit Tm

All drugs used for medical treatment were available as powder and had to be diluted to stock solutions, whereas different concentrations were chosen (Tab. 3). Aliquots were than stored at -20°C.

Drug	Concentration of stock solution
DOX	5 mM
MIMO	5.5 mM
OLO II	100 mM
ROSC	50 mM
TSA	4.4 µg/ml

Tab. 3: List of drug stock solutions and their concentrations

Before each treatment drugs were diluted with adequate medium to a required lower concentrated mastermix and filtered sterile through filters of 0.20 µm thickness. Out of this mastermix all subsequent lower concentrations needed, were diluted with sterile medium. Due to a loss of volume during sterile filtration mastermixes were always prepared with 600 µl of volume additionally.

The drug volume used for treatment depended on the cell culture dishes used and can be seen in Tab. 4.

Cell culture dish	Drug volume used [μ l]
$\varnothing$ 10 cm PD	500
$\varnothing$ 6 cm PD	300
$\varnothing$ 3.5 cm PD	200
well (96 well plate)	100
well (384 well plate)	50

Tab. 4: List of added volumina of drug stock solutions

Experimental conditions regarding drug concentration and treatment period differed in each case, whereas treatment always occurred at the same time.

2.2.3. CellTiter-Glo[®] Luminescent Cell Viability Assay

The CellTiter-Glo[®] Viability Assay is a simple and fast method of determining the number of viable cells after treatment. It is based on the measurement of the ATP level produced by viable cells in culture taking advantage of the luciferase reaction (Fig. 21). After the cells are lysed, ATP released in the supernatant is used by the enzyme luciferase to convert the substrate luciferin to oxiluciferin thereby generating light. The luminescent signal can be measured and is proportional to the ATP level and therefore to the cell number.^[147]

This assay provides information about the proliferation status of cells after treatment and allows to assess the drug cytotoxicity by calculating the IC₅₀. The IC₅₀ is the inhibitory concentration of a drug needed to kill 50 % of all cells.

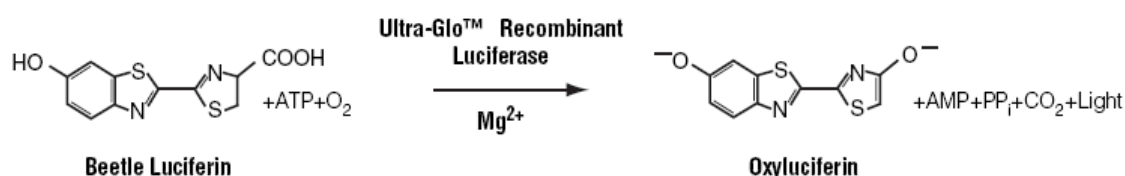


Fig. 21: Luciferase reaction^[147]

Assay Kit

Promega

CellTiter-Glo[®] Buffer

CellTiter-Glo[®] Substrate (lyophilized)

stored at -80°C, thawed and mixed together, stored than at -20°C

After cellular treatment for different incubation times the microtiter plate was centrifuged at 200 x g, 3 min at 20°C to allow cells to accumulate at the subsurface of wells. The medium was reduced to three-quarter of the volume and the buffer-substrate mix was added in a 1:1 ratio. An incubation period of 30 minutes at 37°C (incubator, dark environment) assures cellular lysis and luciferase reaction to take place. The cell suspension was then transferred to a white microtiter plate to avoid light interference from neighbouring adjacent wells and luminescence was measured in a plate reader. ^[147]

2.2.4. Measurement of the DNA content in single cells stained with propidium iodide by flow cytometry

Cells are stained with propidium iodide (PI), a fluorescent dye, for measuring their DNA content in order to determine their cell cycle phase distribution. Propidium iodide intercalates into the DNA strands. Upon excitation DNA-PI complexes generate fluorescence. The intensity of emitted fluorescence is directly proportional to the DNA concentration. It means that cells in G₁ phase have haploid DNA, cells in G₂ phase have diploid DNA and cells with an undefined DNA amount (higher than haploid and lower than diploid) are in S phase. Cells undergoing apoptosis possess partially degraded DNA and generate weaker fluorescence than G₁ cells. They are termed as sub-G₁ or hypoploid cells. ^[148]

For measurement cells are absorbed through a fine capillary tube into the fluorescence-activated cell sorter, where only room for one cell after another (thus generating a single cell suspension is important and essential) exists. Each time a stained cell passes the laser light it scatters the light, its dye is excited to a higher wave length and the cell is counted as one event. Detectors quantify the achieved information. The forward scatter gives information about the cell size, the side scatter about the particle's complexity (nucleus shape, cytoplasmic granulation). The intensity of the emitted fluorescence is proportional to the DNA amount of cells. ^[149]

The flow cytometry studies allow determination of changes in cell cycle phase distribution as well as in rate of apoptosis in response to the used drugs.

Material

10x PBS – 1 L

Dulbecco's phosphate buffered saline (DPBS)
powder without calcium chloride
diluted to 1 L ddH₂O
stored at RT

Sigma

1x PBS – 1 L

10x PBS diluted 1:10 with ddH₂O
stored at 4°C

Trypsin/EDTA		Difco Laboratories
Stock solution (pH 7.6)		
20.4 mM	Tri-Sodium citrate x 2 H ₂ O	Merck
0.6 % (v/v)	NP-40	US. Biochem. Corp.
9 mM	Spermine tetrahydrochloride	Sigma
3 mM	Tris	AppliChem
	stored at -20°C	
Solution A		
9 mg	Trypsin 250 (Difco)	BD
50 ml	stock solution (pH 7.6)	
	stored at -20°C	
Solution B		
150 mg	Chicken Egg White	Sigma
	(type II-0, trypsin inhibitor)	
30 mg	Ribonuclease A	Sigma
	(boiled for 3 minutes before use)	
50 ml	stock solution (pH 7.6)	
	stored at -20°C	
Solution C		
4.9 mg	Propidium iodide	Sigma
69.6 mg	Spermine tetrahydrochloride	Sigma
10 ml	stock solution (pH 7.6)	
	stored at 4°C	

Cells were harvested by limited trypsinization at 37°C for 5 minutes. After inhibition of trypsin activity (using medium) cells were washed twice with 1x PBS for 3 minutes at 900 x g (4°C). The cell pellet was resuspended in 100 µl 1x PBS and transferred to tubes suitable for flow cytometry. To permeabilize cell membranes the single cell suspension was incubated with 75 µl of solution A containing trypsin and incubated for 10 minutes at room temperature. Adding 75 µl of Solution B containing RNase and trypsin inhibitor followed for another 10 minutes. RNase guarantees degradation of RNA (which otherwise would also be stained by propidium iodide) and the trypsin inhibitor stops excessive protein degradation. Finally, cells were stained with 63 µl of Solution C containing propidium iodide. Staining proceeded for at least 30 minutes at 4°C in the dark.^[150] Fluorescence measurement was performed with the flow cytometer Becton Dickinson FACScan. Results are shown as DNA histograms.

2.2.5. Caspase-Glo® 9 Assay

The Caspase-Glo® 9 Assay allows the measurement of caspase-9 activity in treated cells or cellular supernatants. Caspases play a pivotal role in apoptosis and are activated by several environmental stimuli. Caspase-9 is involved in the activation of a whole cleavage cascade, recognizing and cleaving especially the LEHD amino acid sequence (Leu-Glu-His-Asp) of

target proteins, among others pro-caspase-3. As a final consequence of the activation of proteolytic cascade, cells die.

Treated cells are lysed by the assay buffer and the released initiator caspase cleaves the provided substrate, which contains an LEHD sequence. This specific sequence is cleaved and amino-luciferin is generated. The enzyme luciferase uses ATP to convert luciferin to light (Fig. 22). The luminescence generated is proportional to caspase-9 activity. ^[151]

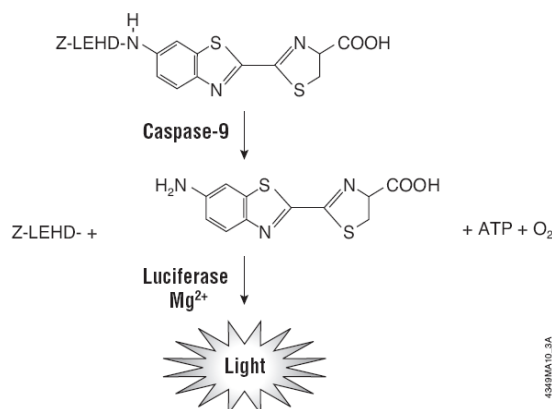


Fig. 22: Caspase-Glo[®] 9 Assay – Cleavage activity of caspase-9 results in luminescence emission by taking advantage of the luciferase reaction ^[151]

Assay Kit

Promega

Caspase-Glo[®] 9 Buffer

Caspase-Glo[®] 9 Substrate (lyophilized)

MG-132 Proteasome Inhibitor

stored at -20°C, thawed and mixed together

Cells are plated into white microtiter plates with a transparent bottom. After a particular treatment period, the microtiter plate was centrifuged at 200 x g, 3 min at 20°C. The medium was reduced to three-quarter of the volume. The buffer-substrate mix was added in a 1:1 ratio and incubated at room temperature in the dark. Luminescence was measured after one and after two hours, ensuring maximal measuring output. Besides untreated controls, medium had to be analysed as well, because of background luminescence. ^[151]

If desired, the supernatant can be transferred to another white microtiter plate and analyzed in the above described manner. At higher drug concentrations majority of cells may die and subsequently release caspases into the surrounding medium. To detect this caspase activity as well, the supernatant may be interesting for analysis.

The recorded relative luminescence units (RLU) during caspase measurement are meaningless until data is normalized to the cell number determined in viability assays. This applies for all performed caspase assays.

2.2.6. Apo-ONE[®] Homogeneous Caspase-3/7 Assay

The Apo-ONE[®] Homogeneous Caspase-3/7 Assay is a good method for detection of caspase-3 and -7 activity. Caspase-3 and -7 recognize and cleave especially the DEVD amino acid sequence (Asp-Glu-Val-Asp) of target proteins (e.g. PARP-1).

Treated cells are lysed by the assay buffer. Released effector caspases can then cleave the provided chemical substrate rhodamine R110 containing the DEVD sequence. The DEVD sequence is cleaved and excitation of rhodamine at 499 nm results in a green fluorescence (emission at 521 nm, Fig. 23). There is a strong correlation between emitted fluorescence and activated caspase-3/7. ^[152]

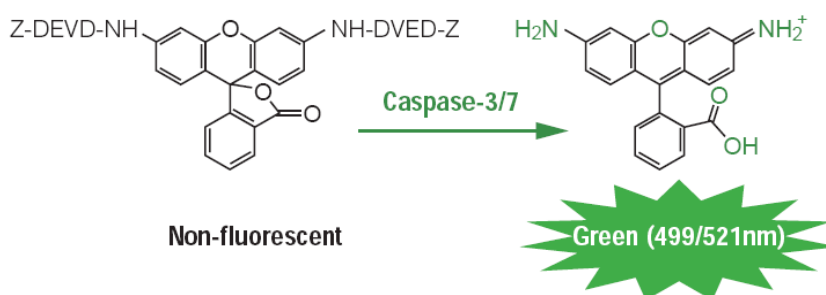


Fig. 23: Apo-ONE[®] assay principle – Cleavage activity of caspase-3/7 results in a fluorescence emission ^[152]

Assay Kit

Promega

Apo-ONE[®] Homogeneous Caspase-3/7 Buffer

1:100 Caspase Substrate Z-DEVD-R110

stored at -20°C, thawed and mixed together

To perform Apo-ONE[®] assay cells were plated in black microtiter plates with a clear bottom. The clear bottom ensures microscopical examination for contaminations, the black plate avoids cross-interference of fluorescent light in adjacent wells.

After a treatment period the microtiter plate was centrifuged at 200 x g, 3 min at 20°C and the medium was reduced to three-quarter of the volume (if desired, the supernatant can be transferred to another black microtiter plate and analyzed in the same manner). The buffer-substrate mix was added in a 1:1 ratio and incubated at 37°C in the dark (CO₂ may disturb the reaction). Fluorescence measuring occurred after 30 minutes, one hour, three hours, six

hours and 18 hours to measure even minimal increase of enzymatic activity. Just in the same way as mentioned before medium had to be measured again. ^[152]

2.2.7. Caspase-Glo[®] 3/7 Assay

<i>Assay Kit</i>	<i>Promega</i>
Caspase-Glo [®] 3/7 Buffer	
Caspase-Glo [®] 3/7 Substrate (lyophilized)	
stored at -20°C, thawed and mixed together	

The Caspase-Glo[®] 3/7 Assay measures like the Apo-ONE[®] Assay the activity of caspase-3/7, but in contrast to this assay the chemical reaction is based on the generation of a luminescent signal (which is more sensitive than fluorescence).

After cell lysis released caspase-3/7 cleaves the provided substrate, whereas the chemical reaction is exactly the same like for the Caspase-Glo[®] 9 Assay, the only difference being in the cleavage sequence recognized (DEVD instead of LEHD). The luminescence generated is proportional to caspase-3/7 activity. The assay procedure is exactly the same as for Caspase-Glo[®] 9 Assay. ^[153]

All assays detecting effector caspases are important tools for detecting ongoing apoptosis after medical treatment. Withal, achieved results can be analyzed for an inversed correlation to viability assays.

2.2.8. 7-Aminoactinomycin D Dye Exclusion Test

7-Aminoactinomycin D, a fluorescent dye similar to propidium iodide, is able to intercalate into DNA strands. Staining cells with 7-AAD is a simple and fast method to distinguish healthy from damaged cells and to quantify the latter. One of the attributes of damaged cells is a disrupted cell membrane, which is an indication for ongoing necrosis. 7-AAD easily passes the disrupted membrane of damaged cells and stains their DNA, whereas viable cells with an intact membrane exclude the dye (Fig. 24). In the cell sorter each cell is excited with the same wave length and analyzed for emitting fluorescence. Thus cells can be classified as fluorescence positive (damaged cells) or negative (healthy cells). Results are evaluated for cell viability and drug cytotoxicity. The higher a drug concentration is the more likely poisoning of cells occurs and cause necrosis. ^[148]

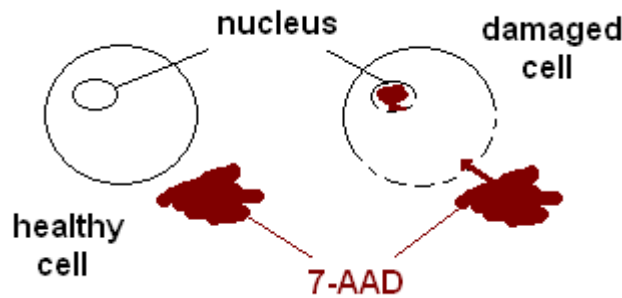


Fig. 24: Dye exclusion test principle using 7-AAD as fluorescent dye

Material

10x PBS – 1 L

Dulbecco's phosphate buffered saline (DPBS)
powder without calcium chloride
diluted to 1 L ddH₂O
stored at RT

Sigma

1x PBS – 1 L

10x PBS diluted 1:10 with ddH₂O
stored at 4°C

Trypsin/EDTA

Difco Laboratories

7-AAD

1 mg	7-AAD
50 µl	abs. EtOH
950 µl	1x PBS

Sigma
Austr. Alco. Österr.

After treatment cellular supernatant was transferred to a Falcon tube and cells were washed with 1x PBS, which was then also transferred to the Falcon. Cells were detached from the underground by adding trypsin and washed twice with 1x PBS at 900 x g, 3 minutes at 4°C. The cell pellet was resuspended in 500 µl 1x PBS and transferred to tubes suitable for flow cytometry. Staining of cells occurs with 5 µl of 7-AAD for 20 minutes at room temperature in the dark. Immediately afterwards flow cytometric measurement has to be carried out. The wave length for excitation is at 555 nm, emission at 655 nm.

2.2.9. In situ M30 CytoDEATH staining

In situ CytoDEATH staining is an immunofluorescence technique for determining caspase-dependent apoptosis in cells. During the switch-on of programmed cell death a couple of effector caspases are activated. They lead to the degradation of several proteins with the purpose to eliminate the cell. Among one of the first cleavage substrates is the cytoplasmic cytokeratin 18, a cytoskeleton protein. A specific antibody (clone M30 CytoDEATH) recognizes and binds to the cleaved cytokeratin product (Fig. 25). The antibody is coupled to the fluorescein (FITC), a green fluorochrom, and can be visualized under the microscope

or alternatively, can be measured by flow cytometry. In healthy cells no cytokeratin cleavage product is generated thus no antibody binding occurs and cells cannot be stained. Positive stained cells show a speckled green fluorescence in the cytoplasm. ^[154]

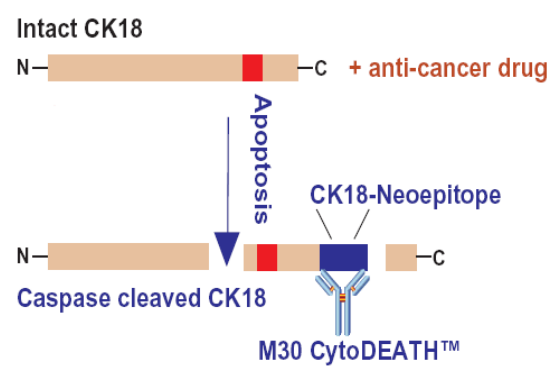


Fig. 25: Cytokeratin 18 cleavage by caspases during apoptosis – M30 CytoDEATH antibody recognizing the cleavage product ^[155]

Material

Lab-Tek® Chamber Slide System (4-well, sterile)	Nunc Internat. Corp.
10x PBS – 1 L Dulbecco's phosphate buffered saline (DPBS) powder without calcium chloride diluted to 1 L ddH ₂ O stored at RT	Sigma
1x PBS – 1 L 10x PBS diluted 1:10 with ddH ₂ O stored at 4°C	
1x PBS-Tween – 1L 10x PBS diluted 1:10 with ddH ₂ O 0.1 % (v/v) Tween-20 stored at 4°C	Sigma
MeOH	Carl Roth GmbH
Triton-X 100	US. Biochem. Corp.
3 % BSA Blocking Solution 1.2 mg BSA 40 ml 1x PBS stored at 4°C	Amresco
1 % BSA Blocking Solution 0.4 mg BSA 40 ml 1x PBS stored at 4°C	Amresco

Cells were plated into a 4-well slide chamber (cell number 5 x 10⁴ cells/ml, 600 µl of cell suspension plated) and treated for indicated periods of time. The medium was discharged and cells were washed twice with 1x PBS. Cells were covered with ice-cold methanol (-20°C) for 30 minutes in order to fix them. Afterwards cells were washed carefully three

times with 1x PBS-Tween. For permeabilization of the cell membrane cells were incubated with 0.2 % Triton-X 100 for 20 minutes. Again washing of cells with 1x PBS-Tween occurred (thrice). Blocking of cells with 3 % BSA/PBS for 30 minutes eliminates unspecific antibody binding and improves the staining quality. Cells were washed thrice with 1x PBS-Tween and then incubated with 150 µl of the M30 CytoDEATH antibody (diluted 1:400 in 1 % BSA/PBS) overnight. Finally, cells were washed three times with 1x PBS and inspected under the microscope. To visualize all cells (not only the apoptotic ones) nuclei were stained with DAPI afterwards.

2.2.10. DAPI staining

DAPI staining represents a typical method for counterstaining in fluorescence microscopy. DAPI is a fluorescent dye binding to the DNA and therefore staining the nucleus. It is excited by ultraviolet light (extinction at 345 nm), displays a blue colour (emission at 455 nm) and passes even intact cell membranes. Hence cells have not to be fixed to be visualized. ^[156]

Material

DAPI stock solution

10 mg	DAPI	DAKO Corporation
2 ml	DMF	Sigma

DAKO® Fluorescent Mounting Medium

DAKO Corporation

10x PBS – 1 L

Dulbecco's phosphate buffered saline (DPBS)	Sigma
powder without calcium chloride	
diluted to 1 L ddH ₂ O	
stored at RT	

1x PBS – 1 L

10x PBS diluted 1:10 with ddH ₂ O
stored at 4°C

DAPI solution is prepared by diluting DAPI stock solution 1:100 in 1x PBS. The obtained solution is further diluted 1:50 in Mounting Medium. DAKO® Fluorescent Mounting Medium prevents a rapid fading of the fluorescence. ^[157]

Fixed or viable cells are covered with DAPI solution for 10 minutes in the dark and washed then with 1x PBS. Cells are kept in 1x PBS to avoid drying-out. Inspection under the microscope can proceed.

2.2.11. Determination of the potential of mitochondrial membrane by JC-1 staining

JC-1 is a cationic, lipophilic dye with fluorescent properties used as a substrate for determination of the integrity of mitochondrial membrane. During early stages of apoptosis its collapse is observed. Due to the release of cytochrome c (a caspase activator) from the mitochondria the electron transport is disrupted and apoptosis is triggered.

JC-1 enters the mitochondria of healthy cells, which usually possess a high membrane potential, and forms J-aggregates of red fluorescence. Apoptotic cells with a low membrane potential fail to accumulate JC-1 in the mitochondria, thus it remains monomeric and emits a green fluorescence.^[158]

Cells can be stained in situ and visualized under a fluorescence microscope or quantified by flow cytometry.

Material

10x PBS – 1 L

Dulbecco's phosphate buffered saline (DPBS)
powder without calcium chloride
diluted to 1 L ddH₂O
stored at RT

Sigma

1x PBS – 1 L

10x PBS diluted 1:10 with ddH₂O
stored at 4°C

10 mM JC-1 Solution

5 mg JC-1
766 µl DMSO

Alexis
Sigma

Trypsin/EDTA

Difco Laboratories

a. In situ staining

Treated cells were washed with warm (37°C) 1x PBS and stained with 20 µM JC-1 for 20 minutes in the dark at 37°C. Thereafter, cells were washed to remove free dye and could then be analyzed under the fluorescence microscope.

b. Staining for flow cytometric measurement

Treated cells were washed carefully with warm 1x PBS and harvested by trypsinization. After three more washing steps (centrifugation at 300 x g, 5 minutes, 20°C) cells were re-suspended in 490 µl warm 1x PBS and transferred to tubes suitable for flowcytometric measurements. JC-1 stock solution (10 mM) was diluted 1:10 with warm 1x PBS. 10 µl (c_E = 20 µM JC-1) of this solution were used to stain the cells for 20 minutes at 37°C in the dark. Ultimately cells were washed four times with warm 1x PBS and analyzed flow cy-

tometrically using two channels: green fluorescence – Ex/Em 510/527 nm, red fluorescence – Ex/Em 585/590 nm. Obtained results are quantitative.

2.2.12. Whole cell lysates

Whole cell lysates are prepared to acquire intracellular proteins for further analyses. Cellular lysis is accomplished by using detergents, which lead to the disruption of the cell membrane without affecting proteins. Cells reject their whole content. Sonification of cells ensures after a centrifugation step the separation of DNA and cell debris from proteins, which are in the supernatant.

Material

10x PBS – 1 L

Dulbecco's phosphate buffered saline (DPBS)	Sigma
powder without calcium chloride	
diluted to 1 L ddH ₂ O	
stored at RT	

1x PBS – 1 L

10x PBS diluted 1:10 with ddH₂O
stored at 4°C

Cell scraper

Sarsted

RIPA buffer

50 mM	Tris/HCl (pH 7.4)	
500 mM	NaCl	Merck
1 % (v/v)	NP-40	US. Biochem. Corp.
0.5 % (w/v)	Na-Doc	Carl Roth GmbH
0.1 % (w/v)	SDS	Carl Roth GmbH
0.05 %	NaN ₃	Sigma
	stored at 4°C	

20mM Pefablock® SC

12 mg	Pefabloc (protease inhibitor)	Merck
50 ml	ddH ₂ O	

0.1 M PMSF

Calbiochem/Sigma

200 mM NaF

200 mM NaVO₃

Stock solution for SDS sample buffer (pH 6.8)

6.006 g	Tris	AppliChem
0.4 g	SDS	Carl Roth GmbH
0.01 g	NaN ₃	Sigma
fill up to 100 ml	ddH ₂ O	
	stored at RT	

2x SDS sample buffer non-stained, non-reduced

0.5 g	SDS	Carl Roth GmbH
1.25 ml	Stock solution for SDS-SB (pH 6.8)	
2.5 mg	EDTA	Merck
5 mg	NaN ₃	Sigma
5 ml	Glycerol	Amresco
fill up to 25 ml	ddH ₂ O	

Cells were scraped off with a cell scraper and washed twice with 1x PBS for 3 minutes at 900 x g. Depending on the pellet size cells were resuspended in between 100 µl and 200 µl RIPA buffer containing the protease inhibitors Pefabloc (1:100) and PMSF (5:100), 200µM natrium vanadate and 200µM natrium fluoride. For some lysates 2x SDS-SB_{non-stained, non-reduced} containing 1:100 PMSF was used to resuspend the cell pellet. Cells were kept on ice for 30 minutes thus making the buffer effective. Afterwards a triple sonification of cells for 10 seconds ensures a DNA free protein suspension after centrifugation at 1500 x g for 5 minutes (cells were kept on ice to avoid heating up). The supernatant was used for further analysis or stored at -20°C.

2.2.13. Cell fractionation - Isolation of nuclei

Cell fractionation is a method for separating cellular components like mitochondria, nuclei and so on. Cells are disrupted with a hypotonic buffer containing ionic and non-ionic detergents and by mechanical pottering. By centrifugation at a higher gravitation nuclei are separated from cytoplasm. In this special case RSB buffer is used to isolate nuclei to study nuclear components.

Material

10x PBS – 1 L

Dulbecco's phosphate buffered saline (DPBS)	Sigma
powder without calcium chloride	
diluted to 1 L ddH ₂ O	
stored at RT	

1x PBS – 1 L

10x PBS diluted 1:10 with ddH₂O
stored at 4°C

RSB buffer

10 mM	Tris/HCl (pH 7.4)	
10 mM	NaCl	Merck
1.5 mM	MgCl ₂	Carl Roth GmbH
stored at 4°C		

10 % Na-Doc

0.1 g	Na-Doc	Carl Roth GmbH
0.9 ml	ddH ₂ O	

10 % NP-40

0.1 ml	NP-40	US. Biochem. Corp.
0.9 ml	ddH ₂ O	

RIPA buffer

50 mM	Tris/HCl (pH 7.4)	
500 mM	NaCl	Merck
1 % (v/v)	NP-40	US. Biochem. Corp.
0.5 % (w/v)	Na-Doc	Carl Roth GmbH
0.1 % (w/v)	SDS	Carl Roth GmbH
0.05 %	NaN ₃	Sigma
stored at 4°C		

After treatment cells were harvested by scrapping them off and washed three times with 1x PBS. Subsequently the cell pellet was resuspended in 2 ml ice-cold (-20°C) RSB buffer (around ten times higher volume than pellet) and cells were left to swell for 15 minutes on ice. Detergents (0.25 % NP-40 and 0.15 % NaDoc) were added slowly to the cells, vortexed quickly and kept 5 more minutes on ice. Cells were pottered approximately 20 times and then centrifuged at 4500 x g for 20 minutes at 20°C. The supernatant representing the cytoplasmic fraction was discarded and stored in Eppendorfer tubes at -20°C. The pellet containing the membrane bound fraction including nuclei was resuspended in RIPA buffer and stored at -20°C as well.

2.2.14. Determination of protein concentration

For a quantitative determination of protein concentration in different samples DC assay from Bio-Rad Laboratories was used, whereas bovine serum albumin (BSA) served as a standard. Standards are usually dilution series of known concentrations of one and the same protein. Their absorbance values are plotted against the corresponding concentration and the generated regression line enables to calculate the concentration of unknown protein samples from the measured absorbance.

The DC assay is a modified Lowry assay based on a two-step colorimetric reaction. Proteins react with an alkaline copper tartrate solution (solution A) to reduce a Folin reagent (solution B) resulting in a blue colour change. Aromatic amino acids like tyrosine and tryptophan (to a less extent cysteine and histidine) are responsible for the colour development. [159]

Quantification of protein concentration in different samples assures that the same protein amount is used for further analysis. This allows comparative statements about protein expression and their modification status (like in for instance treated and untreated cells).

Material

BSA stock solution [50 µg/µl]		
50 mg	BSA	Amresco
1 ml	ddH ₂ O	
Microtiter plate (96-well)		Greiner Bio-One
DC Protein Assay		Bio-Rad
reagent A		
reagent B		

The DC assay was performed in 96-well microtiter plates using following procedure. The BSA standard dilution was prepared as specified, thereby using the corresponding buffer

utilized for preparing the sample (Tab. 5). Each sample was diluted twice with ddH₂O in order to obtain a statistical mean value. The reagents were added (25 µl reagent A and 200 µl reagent B) and after an incubation time of 15 minutes at room temperature absorbance was measured at 750 nm in a plate reader.

Determined protein concentrations have a unit of µg/µl and every protein amount needed can be calculated.

BSA [μg]	Vol. H ₂ O [μl]	Vol. Buffer [μl]	Vol. BSA [2 μg/μl]	Vol. BSA [10 μg/μl]
	4.0	1.0	-	-
	4.0	1.0	-	-
1	3.5	1.0	0.5	-
2	3.0	1.0	1.0	-
3	2.5	1.0	1.5	-
3	2.5	1.0	1.5	-
5	1.0	1.0	2.5	-
7	0.5	1.0	3.5	-
7	0.5	1.0	3.5	-
10	3.0	1.0	-	1.0
15	2.5	1.0	-	1.5
20	2.0	1.0	-	2.0
20	2.0	1.0	-	2.0
25	2.5	1.0	-	2.5
	BSA stock solution [50 μg/μl]			
BSA [10 μg/μl]	1:5	10 μl + 40 μl ddH ₂ O	BSA [10 μg/μl]	
BSA [2 μg/μl]			1:5	10 μl + 40 μl ddH ₂ O

Tab. 5: Protein determination – Pipetting scheme ^[159]

2.2.15. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis is the separation of proteins in an electric field, whereas proteins migrate through a net of acrylamide according to their size. Proteins with a higher molecular weight do not migrate as far as low molecular proteins since they cannot pass a certain acrylamide pore size. The acrylamide pore size is dependent on the used acrylamide concentration and the amount of cross-linkers (APS and TEMED). Varying these specifications allows the analysis of different molecular weight ranges.

To ensure that migration occurs only by size and not by charge, proteins are treated with SDS. SDS denatures the protein's secondary/tertiary structure (at a high temperature), thus facilitating the migration through the gel. Additionally it masks the protein's own charge giving them a uniform negative charge. Proteins migrate that way to the positive charged pole, the anode. Treatment with DTT reduces disulfide bonds also helping in dissolving the protein's structure. Finally the sample is mixed with glycerol, which weighs the sample down to a well of a gel, and with bromphenol blue to visualize it during migration (to avoid running out).

A gel usually consists of an upper stacking gel and a lower separation gel. The stacking gel is composed of a low acrylamide concentration with a large pore size. Its function is to concentrate all proteins at the same front-line for optimal separation. The separation gel has a smaller acrylamide pore size (thus a higher acrylamide concentration) and is used to separate the proteins. ^[160]

Material

Gel cassettes 1.5 mm, Novex	Invitrogen
Acrylamide/bis-Acrylamide 30 % Solution	Sigma
1M Tris/HCl, pH 6.8	
2M Tris/HCl, pH 8.7	
20% SDS	
20 g SDS	Carl Roth GmbH
100 ml ddH ₂ O	
stored at RT	
10 % APS	
1 g APS	Carl Roth GmbH
10 ml ddH ₂ O	
TEMED	Carl Roth GmbH
Combs (10 and 12 wells, 1.00 mm thick)	Invitrogen
2x SDS sample buffer_{stained, reduced}	
0.5 g SDS	Carl Roth GmbH
1.25 ml Stock solution for SDS-SB (pH 6.8)	
2.5 mg EDTA	Merck
5 mg NaN ₃	Sigma
5 mg Bromphenol blue	US. Biochem. Corp.
5 ml Glycerol	Amresco
20 µl 2.6 M DTT	Sigma
BSA/Carbo marker for protein gels (20µl/slot)	
20 µg/µl BSA	Amresco
20 µg/µl Carbonic anhydrase I	Sigma
120µl 2x SDS-SB _{stained/reduced}	
Carbo/Cytochrome C marker for protein gels (20µl/slot)	
20 µg/µl Carbonic anhydrase I	Sigma
20 µg/µl Cytochrome C	
120µl 2x SDS-SB _{stained/reduced}	

10x Electrophoresis buffer – 2 L

288 g Glycine
 60 g Tris
 20 g SDS
 fill up to 2 L ddH₂O
 stored at RT

Carl Roth GmbH
 AppliChem
 Carl Roth GmbH

1x Electrophoresis buffer – 1 L

10x Electrophoresis buffer diluted 1:10 with ddH₂O
 stored at RT

Electrophoresis Chamber, Novex-X-cell II

Invitrogen

First of all separating gels of various acrylamide concentrations were prepared by mixing the required solutions together being careful to add APS and TEMED at last (otherwise the gel would polymerize too fast to be poured). Table 6 is showing the gel ingredients and the mixing instruction.

Separation gel	8 %	10 %	12 %	15 %	Stacking gel	4 %
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
Bidest. H ₂ O	4759.8 µl	4160 µl	3579.8 µl	2710 µl	Bidest. H ₂ O	1753.6 µl
total	8.8 ml	8.8 ml	8.8 ml	8.8 ml	total	2.4 ml

Tab. 6: SDS-PAGE – ingredients for stacking and separation gel ^[161]

The separation gel was poured into the cassette up to three-fourths of its height and overlaid with 1 ml of ddH₂O. It should prevent fissuring of the gel due to oxygen perturbation. After the gel polymerized for approximately 45 minutes the water was removed and the stacking gel was poured above. Insertion of a comb created the wells (10 or 12 wells). The stacking gel polymerized for another 45 minutes and the cassette was then fixed into the electrophoresis chamber, which was filled up with 1x electrophoresis buffer as an electrolyte.

30 µg of proteins were dissolved in reduced and stained 2x SDS-sample buffer to an end volume of 25 µl, boiled up at 95°C for 3 minutes and cooled down on ice. After spinning down samples were loaded onto the gel (markers were used occasionally). Standard run-

ning time was 2 hours at 130 volt. After separation of proteins they were blotted onto a membrane.

2.2.16. Electroblotting (Western Blot)

Electroblotting is the electric transfer of separated proteins from a polyacrylamide gel to a membrane. The electric field is thereby established perpendicular to the gel, whereas the gel has tight contact to the membrane. Thus proteins are able to migrate due to their negative charge to the membrane (placed on anode side). They irreversibly bind due to hydrophobic interactions to the membrane and keep their separation arrangement. This technique enables further protein analysis by antibodies.

Material

MeOH		Carl Roth GmbH
10x Blotting buffer – 2 L		
288 g	Glycine	Carl Roth GmbH
60 g	Tris	AppliChem
4 g	SDS	Carl Roth GmbH
fill up to 2 L	ddH ₂ O	
	stored at RT	
1x Blotting buffer – 2 L		
200 ml	10x Blotting Buffer	
400 ml	Methanol	Carl Roth GmbH
1400 ml	ddH ₂ O	
pH	8.3	
	stored at 4°C	
Hybond-P PVDF membrane, Westran S		Whatman
Nitrocellulose membrane, Optiran Ba-s		Whatman
Sponges for Blotting		
Blotting Chamber, Trans-Blot Cell		Bio-Rad
Cooling system, refrigerated circulator LTD 6G		Grant
Magnetic stirrer		

After running a gel electrophoresis the cassette was broken up to get access to the gel to assemble the blotting sandwich (see Fig. 26 a.). In a gel holder blotting cassette a sponge, soaked in 1x blotting buffer, was placed on the cathode side. The gel was positioned on three Whatman papers on the sponge (right orientation is important!) and the membrane, activated in methanol (or water in the case of nitrocellulose), was placed on top of it. It was a prerequisite to prevent introducing bubbles between the gel and the membrane since they would affect the transfer quality. Again two Whatman papers were carefully put on the membrane and a second sponge completed the sandwich. Every part of the sandwich was

soaked in 1x blotting buffer and kept wet over the whole preparation time. Afterwards the blotting cassette was closed and fixed in the trans-blot cell. The cell was filled up with 1x blotting buffer and connected to a power supply (Fig. 26 b.). Blotting took place at 30 volts over night at 4°C using a cooling system and a magnetic stirrer.

Due to a high binding specificity and mechanical stability PVDF and improved nitrocellulose membranes were used.

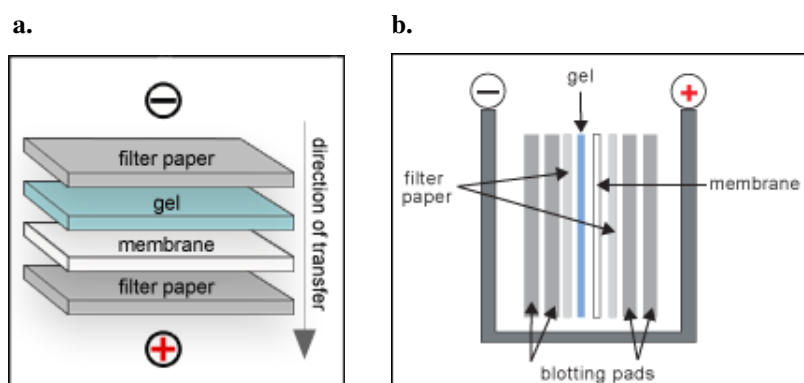


Fig. 26: a. Western Blot assembly, b. Position of gel and membrane regarding the poles in a blotting cell ^[162]

2.2.17. Ponceau S staining

Ponceau S staining is a fast mode of reversibly staining immobilized proteins on a membrane. It does not interfere with the subsequent immunodetection, but it is not quantitative as well. However, the efficiency of blotting and equal protein loading can be checked very easily.

Material

Ponceau S

0.1 % (w/v)	Ponceau S
5 % (v/v)	Acetic acid
fill up to 50 ml	ddH ₂ O

Merck
Fluka Chemika

MeOH

Carl Roth GmbH

After blotting and disassembling the membrane was activated in methanol (water) and stained in Ponceau S solution for 15 minutes. Methanol/Water was used to wash out the surplus of colour and the membrane was dried. Stained membranes were scanned for documentation and either used for immunodetection or stored at room temperature protected from light.

2.2.18. Immunodetection

Immunodetection is a method using specific antibodies to detect a desired protein on a membrane. Therefore a membrane is first incubated with a primary antibody targeting one or several epitopes on the designated protein. Following incubation with a secondary antibody, which specifically recognizes the F_c part of the primary antibody, allows a detection reaction to take place when a substrate is provided. In our case used secondary antibodies were coupled to two different molecules leading to different reactions.

a. Horseradish peroxidase coupled secondary antibody

If the secondary antibody is directly coupled to the enzyme horseradish peroxidase (Fig. 27 a.), it will immediately oxidize luminol (the provided substrate) generating luminescence. The luminescence signal is proportional to the amount of bound primary antibody and the amount of protein present. ^[163]

b. Biotin coupled secondary antibody

In contrast, a secondary antibody can also be linked to the vitamin biotin (vitamin B7, Fig. 27 b.). Streptavidin, a bacterial protein, is coupled to HRP and used to detect biotin. Streptavidin exhibits four binding sites for biotin thus resulting in a high affinity binding and signal amplification. ^[164]

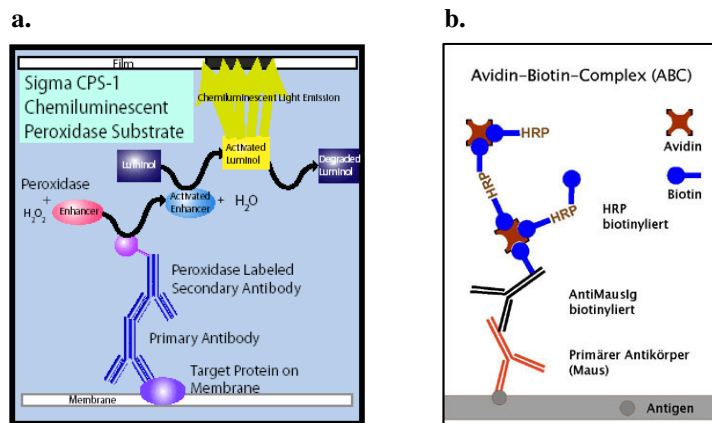


Fig. 27: a. Chemiluminescence reaction of HRP coupled secondary antibodies ^[165], b. Immunodetection using Biotin coupled secondary antibodies and streptavidin ^[166]

To visualize the generated chemiluminescence sensitive autoradiographic films or a Chemi-SmartTM system are used. If utilizing films, the membranes have to be placed in an autoradiograph cassette and overlaid with a film for a chosen exposition time. Protein bands are visible, were antibodies had bound. Chemi-SmartTM on the other hand is a chemiluminescence imaging system allowing a fast autoexposure of membranes with a low

background signal. Images are recorded by a CCD camera and protein bands can be quantified afterwards by the analysing software Bio-1D Advanced.^[167]

Material

10x TBS – 2 L			
28.4 g	Tris		AppliChem
160 g	NaCl		Merck
fill up to 2 L	ddH ₂ O		
pH	7.4 (with HCl)		
	stored at RT		
1x TBS-Tween – 2L			
200 ml	10x TBS		
1800 ml	ddH ₂ O		
2 ml	Tween-20 (0.1 %)		Sigma
	stored at RT		
5 % Milk Blocking Solution			
2.5 mg	milk powder		Fixmilch Instant
50 ml	1x TBS-Tween		
	stored at 4°C		
3 % BSA Blocking Solution			
1.2 mg	BSA		Amresco
40 ml	1x TBS-Tween		
	stored at 4°C		
NaN₃			
ECL Plus Western Blotting Detection Reagents			Amersham Biosc.
25 µl	solution A		
1000 µl	solution B		
Super Signal® West Dura Extended Duration Substrate			Pierce
500 µl	Peroxide Buffer		
500 µl	Luminol Enhancer Solution		
X-Omat Blue XB-1			Kodak

To avoid unspecific antibody reactions with parts of the membrane others than proteins themselves, membranes were saturated for at least 1 hour with 5 % milk (for total proteins) or 3 % bovine serum albumin (BSA) in the case of phosphorylated protein detection after Ponceau S staining. Membranes were then incubated with specific antibodies over night at 4°C on a shaker (except otherwise indicated by company). They are normally diluted 1:1,000 in 3 % BSA solution supplemented with 1:50 NaN₃ to prolong their stability. Primary antibodies were always re-used and stored at 4°C. The membrane was washed four times with 1x TBS-Tween to dispose the excess of antibody (for about 10 minutes). Antibody-antigen complexes were then detected with appropriate secondary antibodies diluted most of the time 1:10,000 (for total proteins) or 1:5,000 to account phosphorylated proteins. Incubation time was 1.5 hours. Afterwards the membrane was properly washed as described before and incubated with the enhanced chemiluminescence detection reagent

ECL-Plus for 3 minutes on a shaker. ECL mix was prepared shortly before applied, according to distributor by diluting the solutions 1:40 (25 µl Solution A mixed with 1 ml solution B). To achieve better signal-noise ratio, the SuperSignal® from Pierce was sometimes used (incubation time: 5 minutes). Signal detection occurs as mentioned previously.

Usually proteins were first checked for their phosphorylation status and then for total protein amount. This allows predictions about their activity and protein expression. Membranes were incubated several times with distinct antibodies of various molecular weights. Ultimately equal protein loading was additionally checked by incubation with antibodies against the housekeeping protein actin or against the GTP binding protein Ran.

2.2.19. Stripping of membranes

Stripping of membranes removes the primary and secondary antibody from a probed membrane thus facilitating successive detection of several proteins on the same blot. Additionally, elimination of unspecific signals can be reduced. Such signals are produced especially when the same secondary antibodies in different sequential detection series are used. However, it is very important to optimize stripping conditions in order not to remove the membrane bound protein, although a small amount will always be lost. This may result in a reduced sensitivity. Nevertheless it is a fast and time saving method. ^[168]

Material

Stripping buffer		
100 mM	DTT	Sigma
2 %	SDS	Carl Roth GmbH
62.5 mM	Tris/HCl (pH 6.7)	
fill up to 100 ml	ddH₂O	
10x TBS – 2 L		
28.4 g	Tris	AppliChem
160 g	NaCl	Merck
fill up to 2 L	ddH ₂ O	
pH	7.4 (with HCl)	
	stored at RT	
1x TBS-Tween – 2L		
200 ml	10x TBS	
1800 ml	ddH ₂ O	
2 ml	Tween-20 (0.1 %)	Sigma
	stored at RT	
3 % BSA Blocking Solution		
1.2 mg	BSA	Amresco
40 ml	1x TBS-Tween	
	stored at 4°C	

Prior to stripping the membrane, it is activated in MeOH and washed twice with 1x TBS-Tween for 10 minutes. Incubation in pre-heated stripping buffer for 20 minutes at 60°C at rotational movement removes the antibody complexes. Afterwards the membrane is washed thrice in 1x TBS-Tween buffer and blocked in 3 % BSA for one hour. The same membrane can then be re-probed with other antibodies.

2.2.20. Statistical evaluation

Software

GraphPad Prism 5

Biostatistical evaluation of achieved results was performed with the GraphPad Prism 5 software. Sigmoidal inhibitory dose-response curves are generated by plotting drug concentrations logarithmically on the x-axis against response values in percent on the y-axis using the nonlinear regression curve. Using row statistics, mean values and standard deviation from all results of the same data set were calculated. Dose-response curves permit the determination of the inhibitory concentration of a specific drug.

Significances of different drug concentrations were analyzed using one-way ANOVA with Dunnett's post test, which compares all columns to the control column. It was presumed that all data show a Gaussian distribution.

For such significance analysis a null hypothesis is assumed to describe a measured data set statistically. The assumption is valid until the data rebuts the hypothesis proving the contrary. It is up to the p-value to characterize how likely the null hypothesis is. The p-value indicates the probability of observing the same (or greater) differences in random samples as the one achieved in a recent experiment. It is always a value between 0 and 1. Values close to 0 invalidate the null hypothesis, that means differences in samples are not random, whereas 1 represents no difference between random samples. The lower a p-value the bigger is the significance of a measured data set, disabling the null hypothesis. A p-value for instance smaller than 0.01 represents the probability of 99 % that occurring differences are not random, but real and a probability of 1 % that differences are random. To be more concrete, in our specific case the null hypothesis is: All populations have identical mean values. Hence, the used drugs are ineffective. If data display small p-values, it is unlikely that observed differences are due to random sampling. The null hypothesis is unlikely, the verified conclusion is that the tested drug is effective. For experiments the chosen significance threshold p-value was 0.05 %. Asterisks in figures represent the estimated significances

due to p-values, whereas $p < 0.001$ corresponds to ***, $p < 0.01$ corresponds to **, $p < 0.05$ corresponds to *.

Statistical evaluation is of great importance to make reliable and real conclusions which are not based on coincidences.^[169, 170]

The diploma thesis is based on results published in two original papers and on a contribution presented during minisymposium during *AACR 100th Annual Meeting 2009* (No. 3862), Denver, Colorado:

1. Józefa Węsierska-Gądek, Andreea Borza, Eva Walzi, Vladimir Krystof, Margarita Maurer, Oxana Komina and Stefanie Wandl. **Outcome of Treatment of Human HeLa Cervical Cancer Cells With Roscovitine Strongly Depends on the Dosage and Cell Cycle Status Prior to the Treatment.** *Journal of Cellular Biochemistry*, 2009, **106**: 937-955.
2. Józefa Węsierska-Gądek, Andreea Borza, Oxana Komina and Margarita Maurer. **Impact of Roscovitine, a Selective CDK Inhibitor, on Cancer Cells: Bifunctionality Increases Its Therapeutic Potential.** *Acta Biochimica Polonica*, 2009, **56** (3): 1-7.
3. Józefa Węsierska-Gądek, Andreea Borza, Eva Walzi, Margarita Maurer, Oxana Komina and Stefanie Wandl. **Dual Action of Roscovitine, a Selective CDK Inhibitor, on Human HeLa Cervical Cancer Cells: Inhibition of Phosphorylation of Cell Cycle Regulators and Repression of HPV-encoded Proteins.** *AACR 100th Annual Meeting 2009* (No. 3862), Denver, Colorado

3. RESULTS

3.1. Effects of ROSC, OLO II and DOX on the proliferation of human HeLa and HTB-31 cervix carcinoma cells

As previously mentioned, roscovitine (ROSC) and olomoucine II (OLO II), relative new pharmacological cyclin-dependent kinase inhibitors, are in the centre of interest due to their high potential in modulating cell cycle in cancer cells. The effect of both inhibitors on two human cervical carcinoma cell lines differing in the p53 tumor suppressor and cell cycle status was examined.

In HeLa cells wt p53 protein is not functional and the G₁ restriction checkpoint is abrogated due to the expression of E6 and E7 viral oncoproteins, respectively. In contrast, HTB-31 cells are not virally infected, but they instead harbour mutations in *TP53* and *RB* tumor suppressor genes, which consequently affect their functionality. In the light of above mentioned facts and considering the circumstance that ROSC and OLO II inhibit primarily CDK2 the question appeared, whether they would be able, if at all, to block cell cycle progression.

To assess the impact of CDK inhibitors on proliferation of HeLa and HTB-31 cells luminescent viability assays were performed. At least three independent experiments, each in quadruplicate were done (exact procedure can be taken from methods part 2.2.3.). Treatment with different drug concentrations permitted the generation of dose-response curves and the determination of IC₅₀ values. Hence, it was possible to analyze, if the displayed effects were relevant/real or not and to compare them to other drugs. Additionally, significant analyses of different drug concentrations were performed. All values in the figures stated below represent mean experimental values, whereas standard deviations are shown as error bars in both directions.

OLO II is structurally very similar to ROSC – they differ in only one hydroxyl group. Therefore it was interesting to compare the action of both inhibitors with each other and additionally with doxorubicin, a strong cytostatic drug. Unlike pharmacological CDK inhibitors, DOX is not selective and exerts high cytotoxicity towards normal and transformed cells.

3.1.1. Anti-proliferative effects of ROSC in HeLa cells are time- and dose-dependent, the cell cycle status prior to the onset of treatment may influence the outcome

To determine the efficacy of ROSC several experiments were performed in parallel. Scheme of treatment of HeLa cells with different ROSC concentrations was constant. However, other parameters like the duration of continuous treatment, time period between plating and the start of treatment as well as post-incubation after treatment in a drug-free medium varied. This experimental approach allowed to evaluate not only direct action of the drugs, but additionally at the same dosage their long-term effects. Viability assays were performed immediately after treatment.

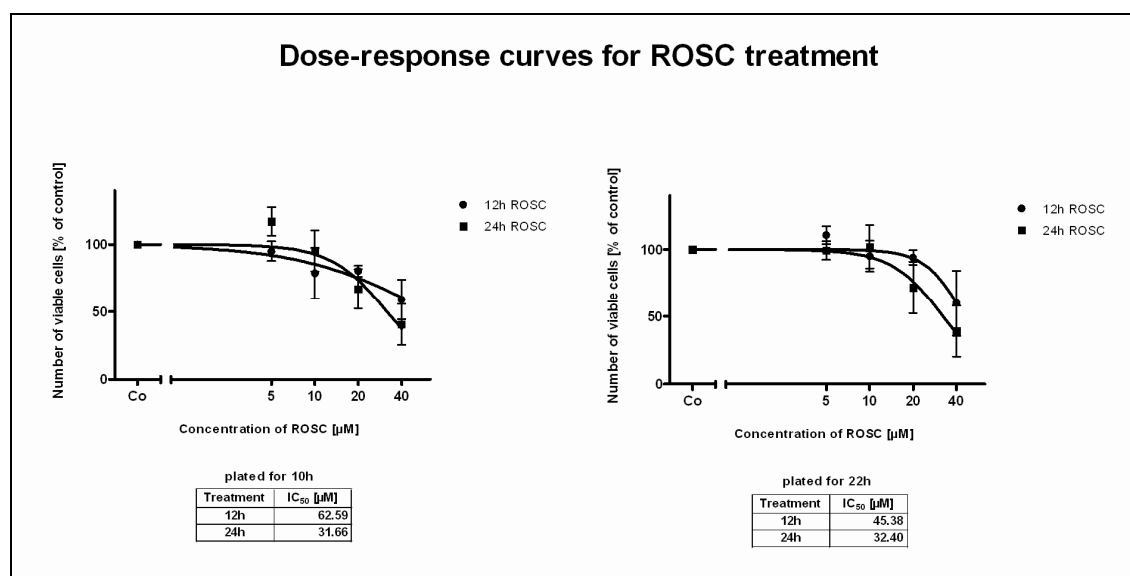


Fig. 28: Dose-response curves for ROSC. Exponentially growing HeLa cells were plated in 96-well micro-titer plates and let to settle down for either 10h (left panel) or 22h (right panel) prior to the onset of treatment. Exposure of cells to ROSC for 12h or 24h clearly reduced the number of viable cells in comparison to untreated controls.

ROSC inhibits proliferation of HeLa cells in a time- and dose-dependent manner (shown in Fig. 28).^[Paper No. 1] As the ROSC concentration increases, the cell number decreases visibly. Lower ROSC concentrations (starting at 10 µM) are able to reduce the cell number, although the reduction is statistically not significant. A reduction of the cell number observed after 12h of the action of 40 µM ROSC is in contrast highly significant and occurs already after treatment in both cases. The observed effect is even stronger after 24h, if IC₅₀ values are considered. Treatment of 24h reveals an inhibitory concentration of about 32 µM for both dose-response curves. However, a comparison between the left and the right panel in Fig. 28 demonstrates differences between the efficacy of treatment depending on

the period after plating that precedes the onset of treatment, especially for the 12h treatment at lower ROSC concentrations.

Thus, above results show lower sensitivity of HeLa cells to medications at 10h after plating as compared to cells at 22h post-plating and indicate that human HeLa cells need a recovery period of approximately 20h prior to the administration of drugs. Therefore, based on these observations, we decided to start the treatment of cells in all subsequent experiments only after full recovery period.

3.1.2. Long-term effects of ROSC

In the next experiments the continuous and discontinuous treatment modi were compared. Since experiments showed that ROSC definitely acts over time, longer treatment periods become of great interest. Cells were exposed to ROSC for 24h and 48h or, alternatively, cells were treated for 24h and then after a medium change (MC) post-incubated in a drug-free medium for the next 24h.

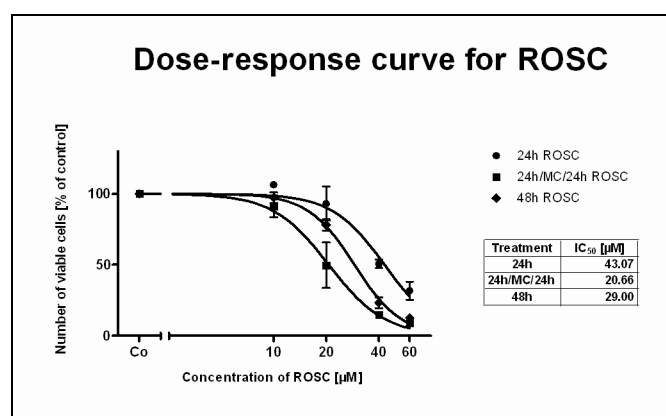


Fig. 29: Dose-response curve for ROSC upon varying the exposure time. HeLa cells were plated in 96-well microtiter plates. After a 20h recovery period before the onset of treatment cells were incubated with different ROSC concentrations for 24h or 48h. Alternatively, after ROSC treatment for 24h the medium was changed and cells were post-incubated for the further 24h in a drug-free medium.

According to expectations treatment of HeLa cells for 48h succeeded in reducing the cell number at much lower concentrations when compared to the short-term incubation (Fig. 29).^[Paper No. 2] Surprisingly, a medium change after 24h of ROSC treatment is even more potent than the continuous treatment itself. Comparison of IC₅₀ values evidenced the difference between each treatment. At the same dosage, the post-incubation of treated cells increased the anti-proliferative efficiency by more than 50 % (43 vs. 20 μM ROSC). This indicates that this kind of treatment reflecting the effect of the drug after *in vivo* administration seems to be very effective and quite promising for the near future.

3.1.3. Higher ROSC concentrations are statistically highly significant

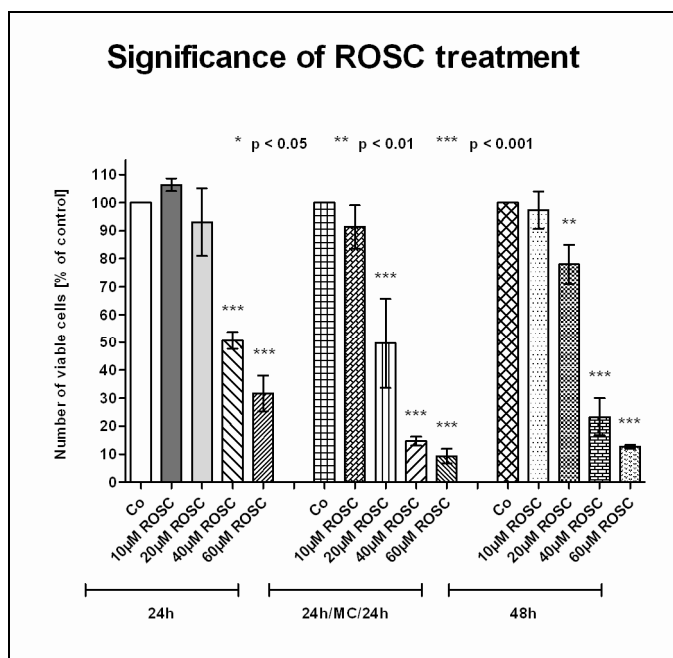


Fig. 30: Statistical significances of the reduction of the number of living cells after ROSC treatment.

To evaluate which ROSC concentration reduced the proliferation of HeLa cells at a relevant grade, statistical analyses were performed. The effect of different ROSC concentrations was compared to that of untreated cells, considering thereby various incubation times. Statistical analyses was performed using one-way ANOVA with Dunnett's post test.

Statistical analyses indicate that ROSC at higher concentrations (40 μ M and 60 μ M ROSC) exhibit highly significant anti-proliferative effects ($p < 0.001$) observed directly after treatment for 24h, 48h and after 24h with a 24h post-incubation period (Fig. 30). [Paper No. 1] Remarkably, the 24h treatment at 20 μ M ROSC followed by a post-incubation in a drug-free medium is very significant despite a relatively high SD value. The variation of measurement values seems to be attributable to varying adherence of treated cells to the substratum (bottom of plates).

3.1.4. OLO II is a more potent CDK inhibitor than ROSC

In order to compare the effectiveness of OLO II with that of ROSC, HeLa cells were treated with OLO II in the same experimental modus.

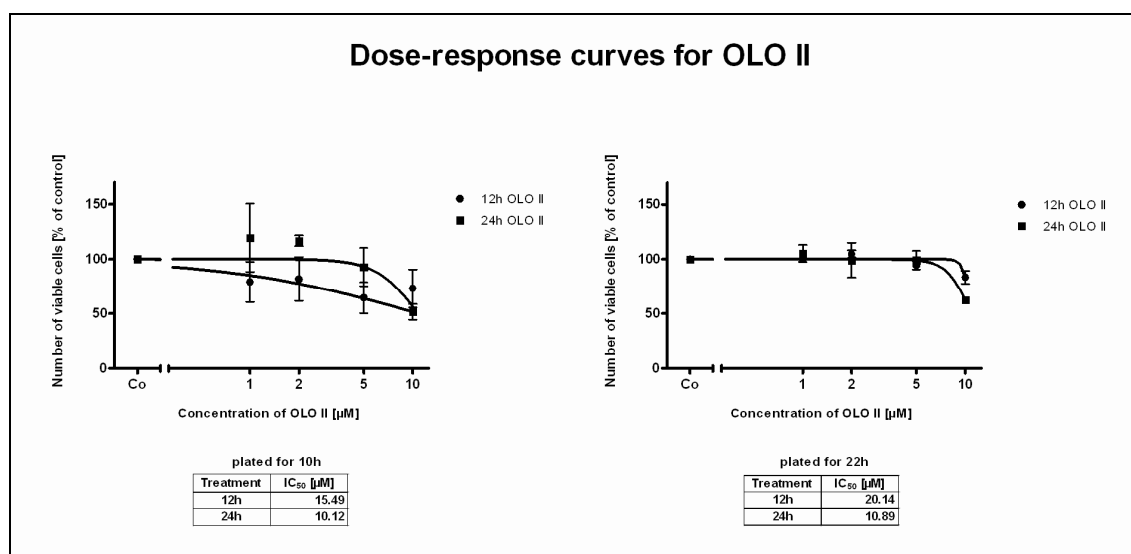


Fig. 31: Dose-response curves for OLO II. Exponentially growing HeLa cells were plated into 96-well microtiter plates and let to settle down for either 10h (left panel) or 22h (right panel) before the onset of treatment. OLO II was administered at different concentrations for 12h and 24h respectively.

OLO II inhibits proliferation of HeLa cells in a time- and dose-dependent manner (Fig. 31). The marked reduction of the cell number occurred at low doses thereby indicating that OLO II is a very efficient agent. In comparison to ROSC similar inhibitory effects were observed at an approximately four-fold lower concentration. Thus the IC₅₀ of OLO II averages 10 µM after 24h. It means that lower OLO II concentrations are sufficient to induce exactly the same effects as achieved by ROSC. Even the treatment with OLO II after 12h seems to be effective. OLO II at lower concentrations, however (1 µM and 2 µM OLO II, respectively), does not affect the cell number. Taken together OLO II is a more potent anti-proliferative CDK inhibitor than ROSC.

Differences in the sensitivity of cells to OLO II depending on the length of period after plating that precedes the onset of treatment, especially for the 12h treatment, were observed. This result is consistent with previous data on ROSC and indicates that HeLa cells need a longer period to recover from stress induced by procedures connected with plating (trypsinization, centrifugations etc.).

3.1.5. Time course of OLO II action

As mentioned, OLO II acts like ROSC in a time- and dose-dependent manner. Experiments were designed to analyze the course of time of OLO II action, investigating a 24h and 48h continuous treatment as well as the 24h treatment with a post-incubation phase for an additional day.

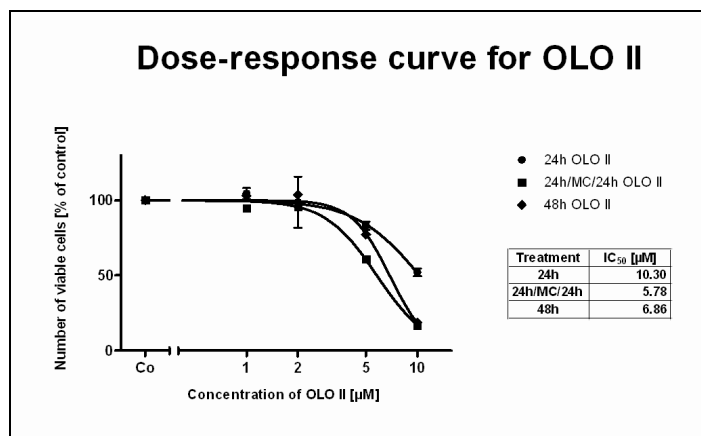


Fig. 32: Dose-response curve for OLO II after varying the exposure time. HeLa cells were plated into 96-well microtiter plates. After a 20h recovery period OLO II was added to final concentrations as indicated. Cells were incubated with the drug for 24h or 48h, respectively. Alternatively, after treatment with OLO II for 24h the medium was changed and cells were post-incubated for the further 24h in a drug-free medium.

Our results revealed the same trend like after ROSC treatment (Fig. 32). The most efficient reduction of the cell number was observed after treatment for 24h with a subsequent medium change and post-incubation for 24h.

The drug concentration sufficient for the 50 % inhibition of cell proliferation in the treatment modus with the post-incubation phase was approximately half (6 μM) of that after 24h treatment.

In conclusion, both CDK inhibitors (ROSC and OLO II) have delayed effects in cells, suggesting that a block of cell cycle or alternatively other processes persist for 24h even in the absence of the drug.

Statistical analyses prove significance of the obtained results: only effects at higher OLO II concentrations (5 μM and 10 μM) are statistically relevant in each approach. At lower drug concentrations nearly all cells (about 96 %) were still viable (Fig. 33).

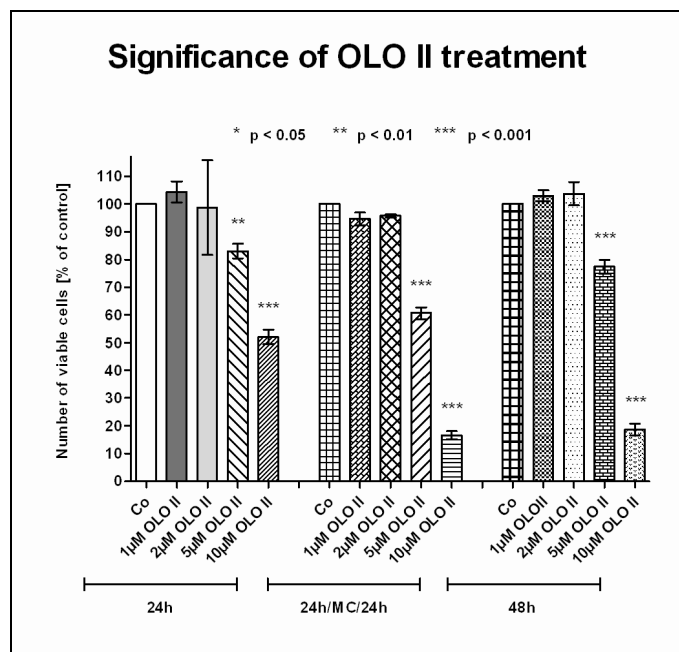


Fig. 33: Statistical significances of the diminution of the number of viable cells upon treatment by OLO II. To evaluate the effect of different OLO II concentrations on the number of viable cells, statistical analyses using one-way ANOVA with Dunnett's post test were performed.

3.1.6. Anti-proliferative effects of ROSC and OLO II as compared to effects exerted by DOX, a highly cytotoxic drug

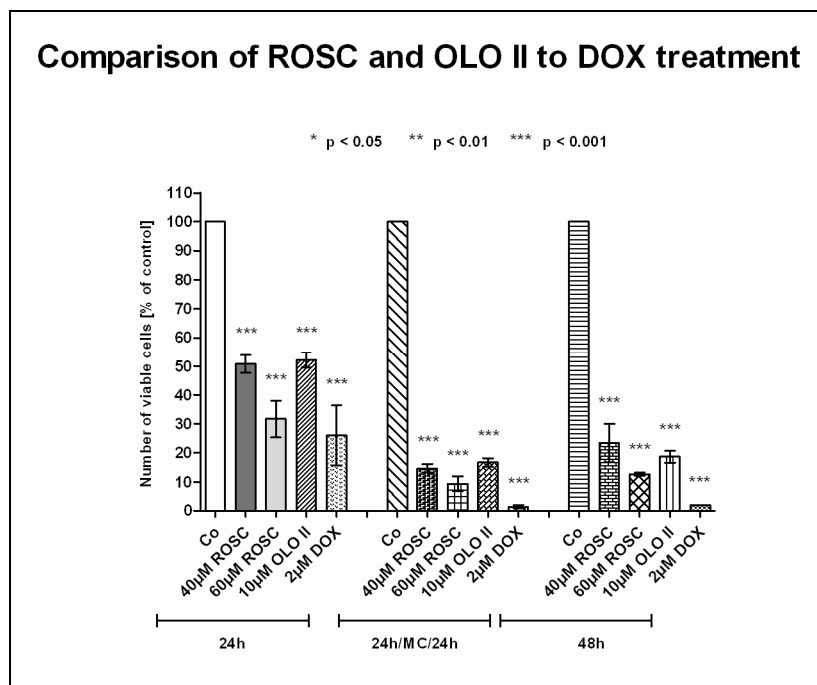


Fig. 34: Comparison of treatment of HeLa cells with ROSC, OLO II and DOX. In addition to ROSC and OLO II HeLa cells were also treated with DOX for the indicated periods of time. Proliferation assays were performed as described earlier. Statistical analyses were performed using one-way ANOVA with Dunnett's post test.

DOX is known to be a highly cytotoxic drug and was used in the present experiments as a control for the treatment with CDK inhibitors.

Figure 34 shows the tremendous power of DOX. After treatment for 24h DOX at low dose (2 μ M) is able to reduce the cell number to approximately a quarter. This effect is strongly enhanced after longer treatment (48h or 24h/MC/24h). Only 1 % of all cells remained viable (Tab. 7). Hence, DOX is, without any doubt, a highly cytotoxic agent acting very efficiently already at low doses.

Unlike DOX, tested pharmacological CDK inhibitors are not so effective. However, it should be underlined that DOX is cyto- and genotoxic.

Our results show that both tested pharmacological CDK inhibitors exerted delayed effects. The post-incubation of cells treated in the absence of drugs for 24h was more efficient than continuous treatment for 48h. Taken together, all obtained results are statistically highly significant.

	Mean of the number of viable cells [% of control]		
	24h	24h/MC/24h	48h
Co	100.0	100.0	100.0
40 μM ROSC	52.0	15.0	23.0
60 μM ROSC	32.0	9.0	13.0
10 μM OLO II	52.0	17.0	19.0
2 μM DOX	26.0	1.0	2.0

Tab. 7: Reduction of the number of viable cells after treatment with examined drugs.

3.1.7. HTB-31 cells are less susceptible to ROSC treatment

Due to the promising effects achieved by ROSC in HeLa cells, investigation of HTB-31, a human cervical carcinoma cell line, regarding the same treatment as in HeLa cells took place.

Experiments were performed according to the same experimental protocol as for HeLa cells.

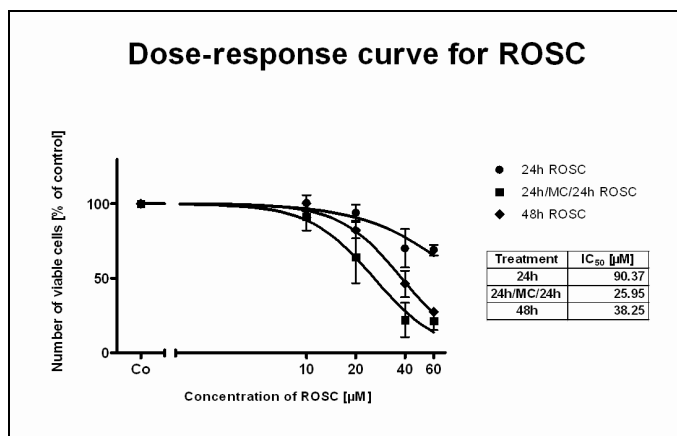


Fig. 35: Dose-response curve for the action of ROSC in HTB-31 cells. HTB-31 cells were seeded 20h before the onset of treatment. The same ROSC concentrations as for HeLa cells were applied to HTB-31 cells for the indicated periods of time. Subsequently CellTiter-Glo® Luminescent cell viability assays were performed as described earlier.

In contrast to HeLa cells, HTB-31 cells are more resistant to ROSC treatment. After treatment for 24h a much higher ROSC concentration (90.37 μM) was needed to inhibit cellular proliferation by 50 %, this constitutes the two- or three-fold dose needed for HeLa cells (Fig. 35). Even a medium change or a treatment for a longer period did not diminish the cell number as efficient as in HeLa cells, although 40 μM and 60 μM ROSC reduced the cell number with a high significance (data not shown).

Post-incubation of treated HTB-31 cells in a drug-free medium was also more efficient than continuous treatment for 48h. This result is of great importance because this mode of treatment mimics the action of drugs *in vivo* administered to patients.

3.1.8. OLO II affects HTB-31 cells more strongly than ROSC

Furthermore HTB-31 cells were exposed to OLO II.

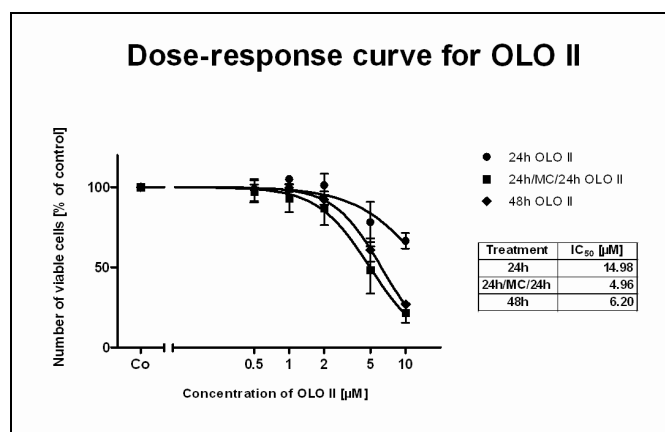


Fig. 36: Dose-response curve for OLO II in HTB-31 cells. HTB-31 cells were treated with different OLO II concentrations as indicated.

A 24h treatment of HTB-31 cells with OLO II is not as potent as in HeLa cells (Fig. 36). The inhibitory concentration needed to reduce the number of living cells by 50 % averaged about 15 μ M. Surprisingly, both long-term treatments (continuous and discontinuous) are in HTB-31 cells as effective as in HeLa cells: showing nearly the same IC₅₀. In the case of the treatment with the post-incubation phase OLO II at a final concentration of 5 μ M is sufficient to reduce the cellular proliferation by 50 %.

OLO II at a final concentration of 10 μ M reduces the cell number to 20 % when it acts longer than 24h (Fig. 37). This substantiates that OLO II is a generally more potent agent and its effect is independent of the cell line.

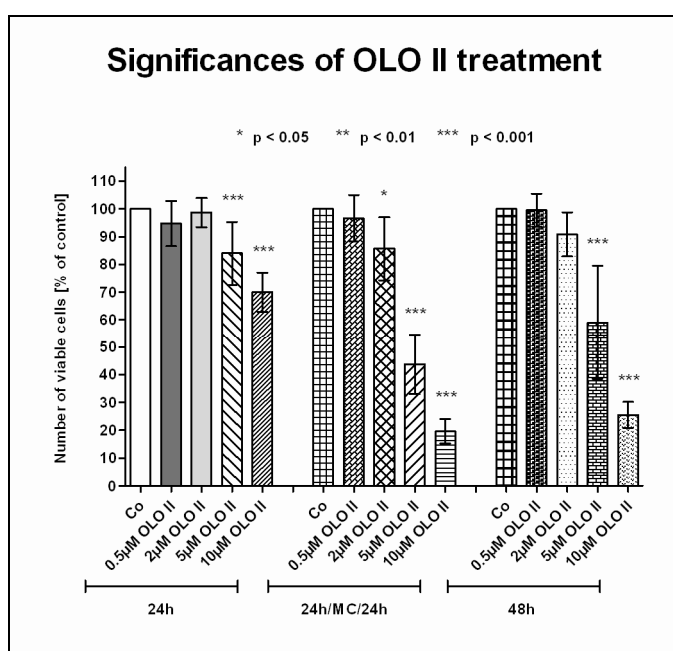


Fig. 37: Significances of OLO II treatment of HTB-31 cells. Statistical analyses of the effects of OLO II treatment on the number of living human HTB-31 cells were performed using one-way ANOVA with Dunnett's post test.

3.1.9. DOX treatment is not so effective in HTB-31 cells

Finally, it was interesting to examine the effect of DOX on HTB-31 cells.

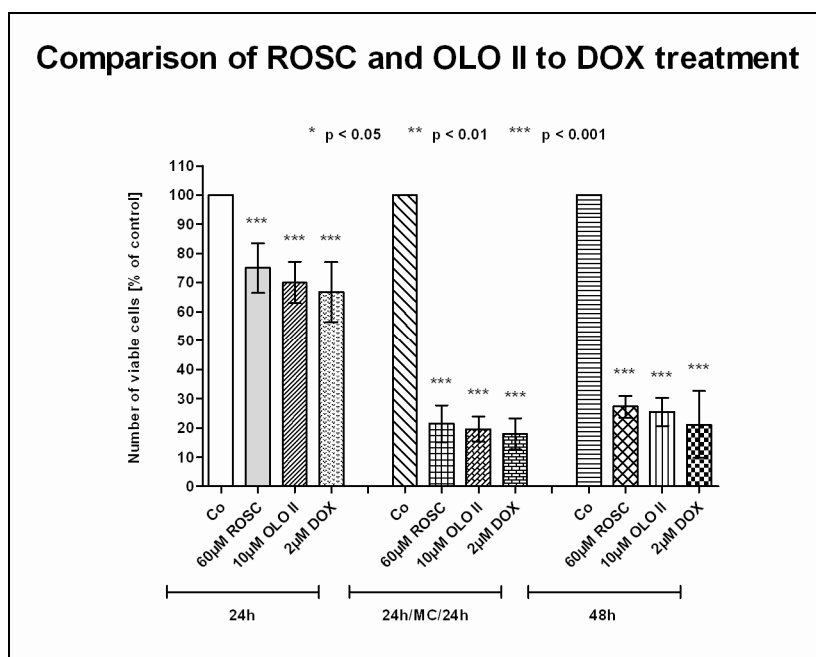


Fig. 38: Comparison of the efficacy of the ROSC and OLO II action with that of DOX in human HTB-31 cells. HTB-31 cells were treated with ROSC, OLO II and DOX as indicated. Proliferation assays were performed as for the other treatments described earlier. Statistical analyses were performed using one-way ANOVA with Dunnett's post test.

As depicted in Fig. 38, HTB-31 cells are less sensitive to DOX than HeLa cells. Even after longer treatment periods about 11 % of cells remain viable (compared to 1 % in HeLa cells). Nevertheless, DOX is still more cytotoxic than ROSC and OLO II. If maybe higher concentrations of CDK inhibitors were chosen, the same effect could be hit. However, this was not further tested, since DOX only served as a control drug.

HTB-31 cells seem to be generally more resistant to the action of conventional cytostatics and CDK inhibitors.

3.2. Effects of ROSC and OLO II on the cell cycle progression in HeLa and HTB-31 cancer cells

ROSC and OLO II strongly affected the cellular proliferation in both examined cancer cell lines. In the next step their action on the cell cycle was examined.

Asynchronously growing HeLa and HTB-31 cells were treated either with ROSC or OLO II at two concentrations and stained with propidium iodide to measure their DNA content by flow cytometry (see propidium iodide staining procedure at 2.2.4.). The generated DNA profiles provided good information about the distribution of cells in G₁, S and G₂/M phase

of the cell cycle. Moreover, the ratio of cells containing DNA concentration lower than cells in G₁ phase, so-called hypoploid or sub-G₁ cells, were also shown. Sub-G₁ cells represent usually apoptotic cells.

At each time point control cells were analyzed in order to monitor spontaneous changes in the distribution of cells in distinct cell cycle phases.

At least three independent experiments were performed. DNA histograms were chosen to illustrate a representative experiment. Moreover, results of at least three independent experiments were additionally shown. Bar graphs showing mean values $\pm$ SD were generated using the GraphPad Prism 5 software.

3.2.1. ROSC induces a G₂/M arrest in HeLa cells accompanied by a high apoptosis rate

Results from proliferation assays showed that ROSC only at higher concentrations is able to efficiently reduce the cell number. Therefore cells exposed to higher ROSC concentrations (20 and 40 μ M ROSC) were analyzed by flow cytometry.

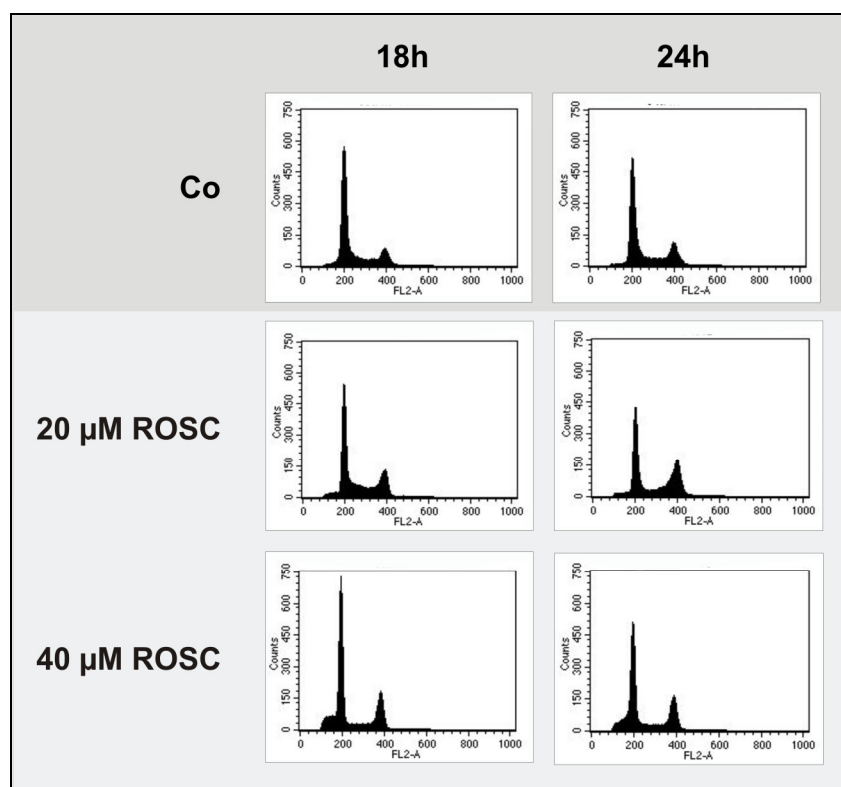


Fig. 39: DNA profiles of control and ROSC-treated HeLa cells

As shown in Figs. 39 and 40, the effect of ROSC on the cell cycle was concentration-dependent. [Paper No. 1] At lower ROSC concentration (20 μ M) increased the frequency of the

G₂/M population and concomitantly diminished that of G₁ phase. After 18h of treatment a transient increase of S phase was observed. Six hours later the G₂/M population reached its highest value of 32.7 %, clearly representing a G₂/M arrest. Concomitantly, a population of sub-G₁ cells appeared (10 %).

ROSC at a twofold dosis resulted in a stronger increase of G₂/M cells appearing already after 12 hours of treatment and was accompanied by a reduction of S phase. Remarkably, the G₁ population remained almost unaffected and was nearly the same as in untreated cells. Moreover, the frequency of hypoploid cells representing cells undergoing apoptosis constituted approximately 20 % of all diploid cells after 12h and increased to 30 % after the next 12 hours. DNA profiles shown in Fig. 39 definitely visualize both effects in response to ROSC treatment: increase of G₂/M cell population and sub-G₁ cells.

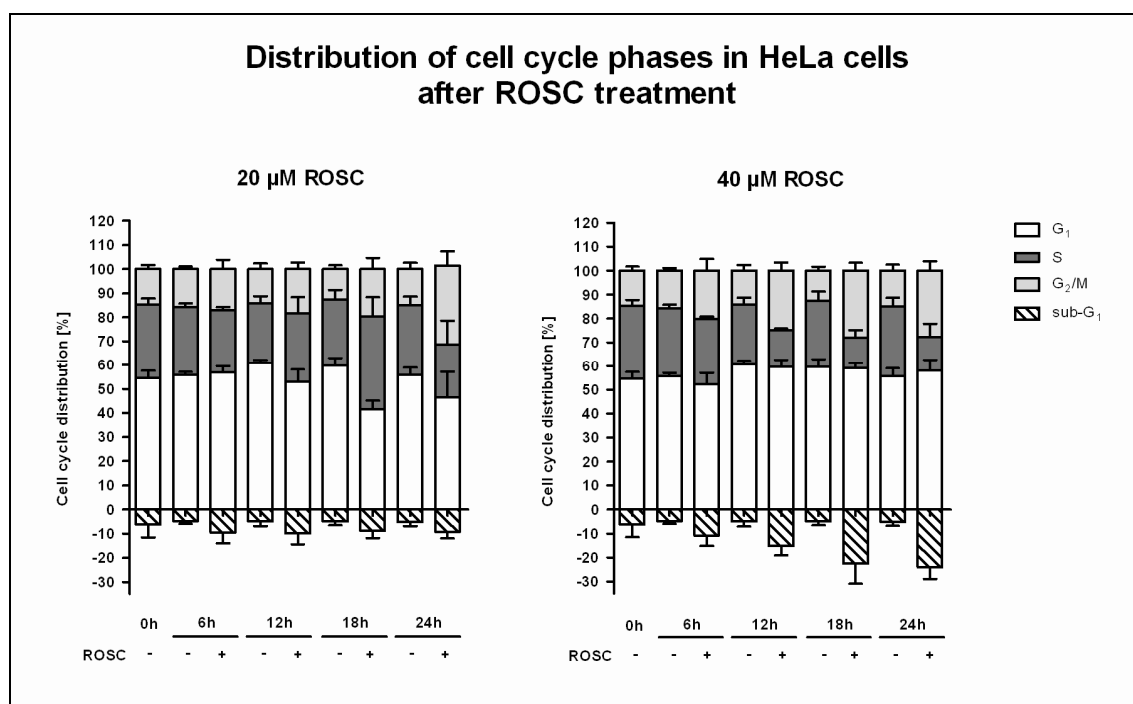


Fig. 40: Distribution of cell cycle phases in HeLa cells after ROSC treatment. HeLa cells were treated with 20 μM or 40 μM ROSC for increasing periods of time, as indicated. After trypsinization of cells, the generated single cell suspension was stained with propidium iodide. DNA content was measured in the flow cytometer.

3.2.2. OLO II arrests HeLa cells at G₂/M at low dosis

Effect of OLO II on the cell cycle of HeLa cells was also investigated. Cells were exposed to 5 μM and 10 μM OLO II for 12h and 24h.

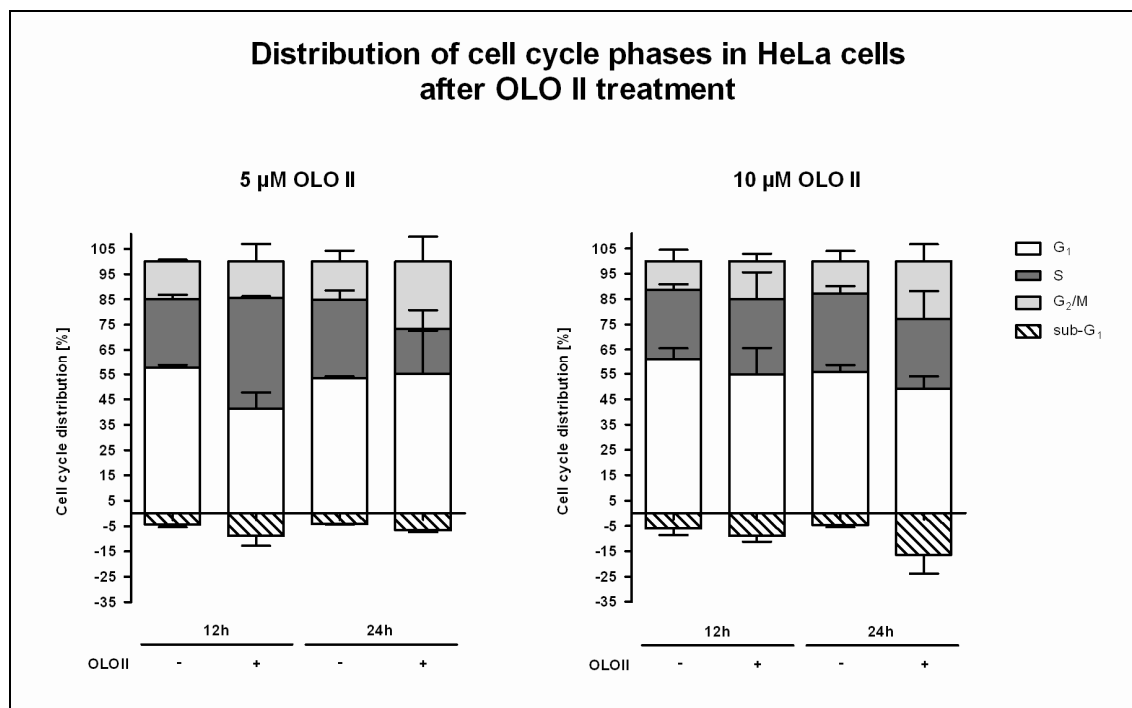


Fig. 41: Distribution of cell cycle phases in HeLa cells after OLO II treatment. HeLa cells were treated with 5 μM or 10 μM OLO II for 12h and 24h. Cells prepared as a single cell suspension were stained with propidium iodide and DNA content was measured by flow cytometry.

At a final concentration of 5 μM, OLO II induced after 12h an accumulation of cells in S phase and a reduction of the G₁ cell population. These changes were transient (Fig. 41). Surprisingly, the frequency of cells in G₂ phase remained unchanged at this time point. After treatment for 24h an increase of G₂/M cells (~ 25 %) associated with a reduction of S phase cells was observed.

In contrast, the effect of OLO II at the twofold dosis on HeLa cells becomes evident after treatment for 24h. Cells were arrested in G₂/M phase and apoptosis at a moderate rate appeared. In summary, OLO II modulates the cell cycle progression of HeLa cells in a similar way and with the comparable efficiency as ROSC, but at much lower concentrations.

3.2.3. ROSC rapidly arrests HTB-31 cells in G₂/M phase but pro-apoptotic effects are delayed

Proliferation assays revealed that HTB-31 cells display a lower susceptibility to ROSC treatment. Therefore experimental protocol in this case was set up in a manner which allowed the monitoring of the cell cycle over a longer period of time. At first cells were stained with propidium iodide every 6h and later every 12h up to two days.

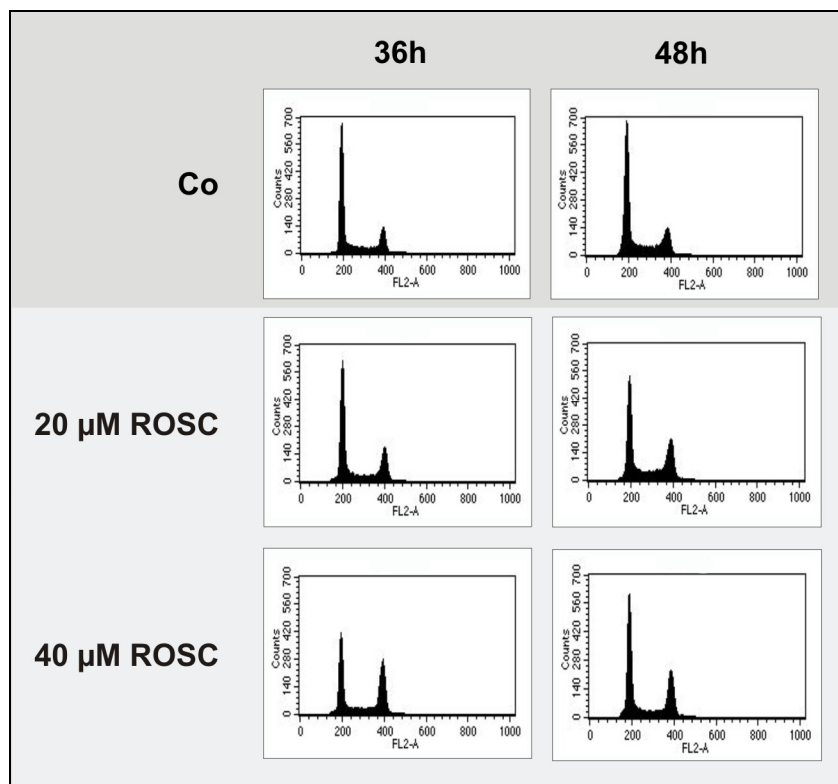


Fig. 42: DNA profiles of HTB-31 cells treated with ROSC for 36h and 48h. The Co showed in the left panel (36h) was obtained from cells at 24h.

Treatment of HTB-31 cells with 20 μM ROSC leads to the accumulation of cells in G_2 phase already after 12h, reaching its highest value of about 30 % after 24h of treatment (Fig. 42). These effects were similar to those observed for HeLa cells.

The ratio of G_2 phase cell population slightly decreased after 36h and 48h of treatment. Concomitantly, the frequency of G_1 cell population was reduced beginning with 18h. Interestingly, ROSC at a final concentration of 20 μM did not induce cell apoptosis.

When treated with 40 μM ROSC, HTB-31 cells arrested stronger in G_2/M phase. After 36h an increase to almost 40 % of G_2 phase occurred (very impressive shown in Fig. 42). [Paper No. 1] This seemed to be at the expense of G_1 cells. Exposure of HTB-31 cells to 40 μM ROSC for the further 12h resulted in diminution of S phase cells and an appearance of hypoploid cells. The flow cytometric analyses indicate that onset of apoptotic response to ROSC is much weaker and delayed than in HeLa cells. Moreover, it proceeds very slowly. The ration of sub- G_1 cells appeared at 36h and increased only by approximately 5 % during the next 12h.

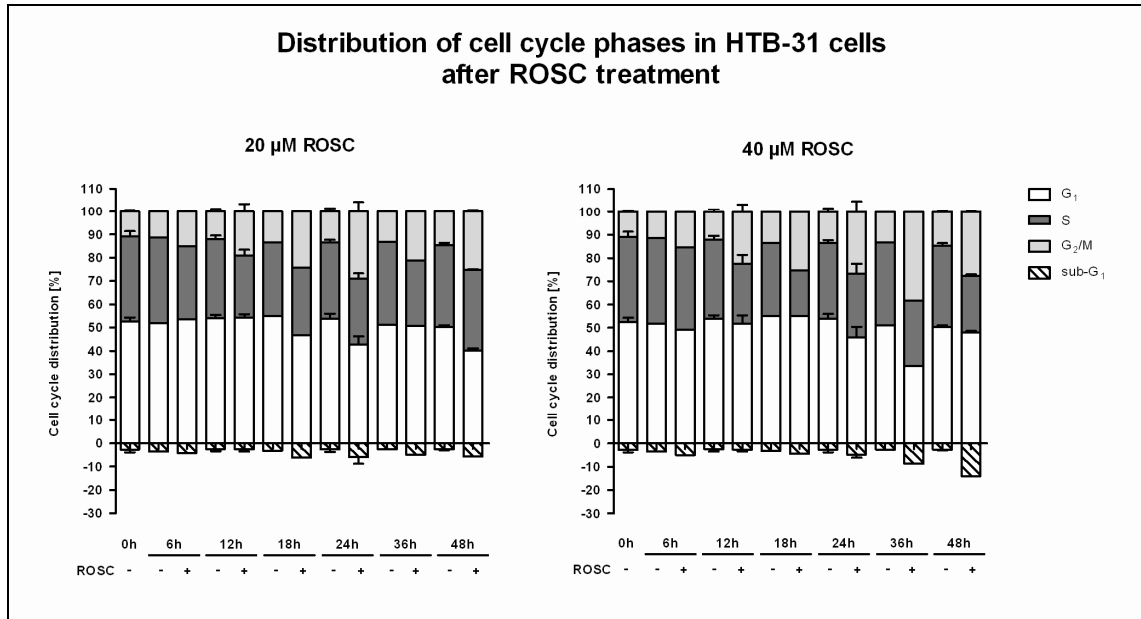


Fig. 43: Distribution of HTB-31 cells in distinct cell cycle phases after ROSC treatment. HTB-31 cells were treated with 20 μ M or 40 μ M ROSC up to 48h, as indicated. After trypsinization of cells, the generated single cell suspension was stained with propidium iodide. DNA content was measured in the flow cytometer. (Statistical data were not obtained for each time point)

3.2.4. Increase of the ratio of G₂/M cell population after OLO II treatment

The next experiments were performed to determine the effect of OLO II on cell cycle progression in HTB-31 cells.

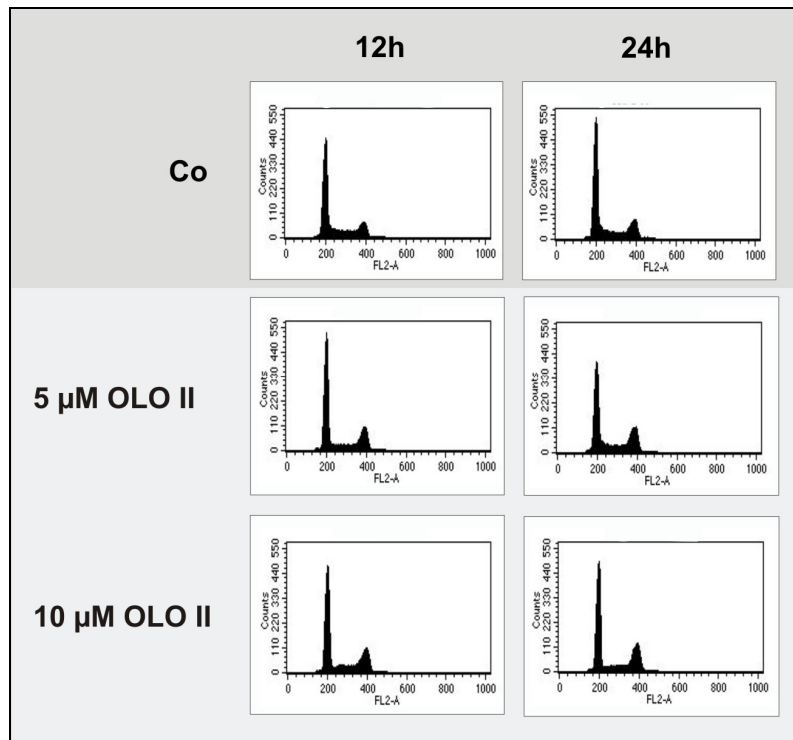


Fig. 44: DNA profiles of control HTB-31 cells and cells treated with OLO II for the indicated time points.

It is not surprising at all that OLO II is also able to induce accumulation of cells in G₂/M phase in HTB-31 cells (Figs. 44 and 45). OLO II at both concentrations (5 μ M and 10 μ M OLO II) induced the G₂/M arrest with the same efficiency as ROSC starting at 12h. The lower concentration increased the G₂/M phase at the expense of G₁ cells, leaving the S phase unchanged as in controls after 24h of treatment. In contrast, 10 μ M OLO II induced the G₂/M arrest at the account of S phase cells.

OLO II did not induce apoptosis in HTB-31 cells within 24h. Considering the fact that HTB-31 cells were relatively resistant to triggering apoptosis by ROSC, it cannot be excluded that after treatment for the further 24h apoptosis could be induced.

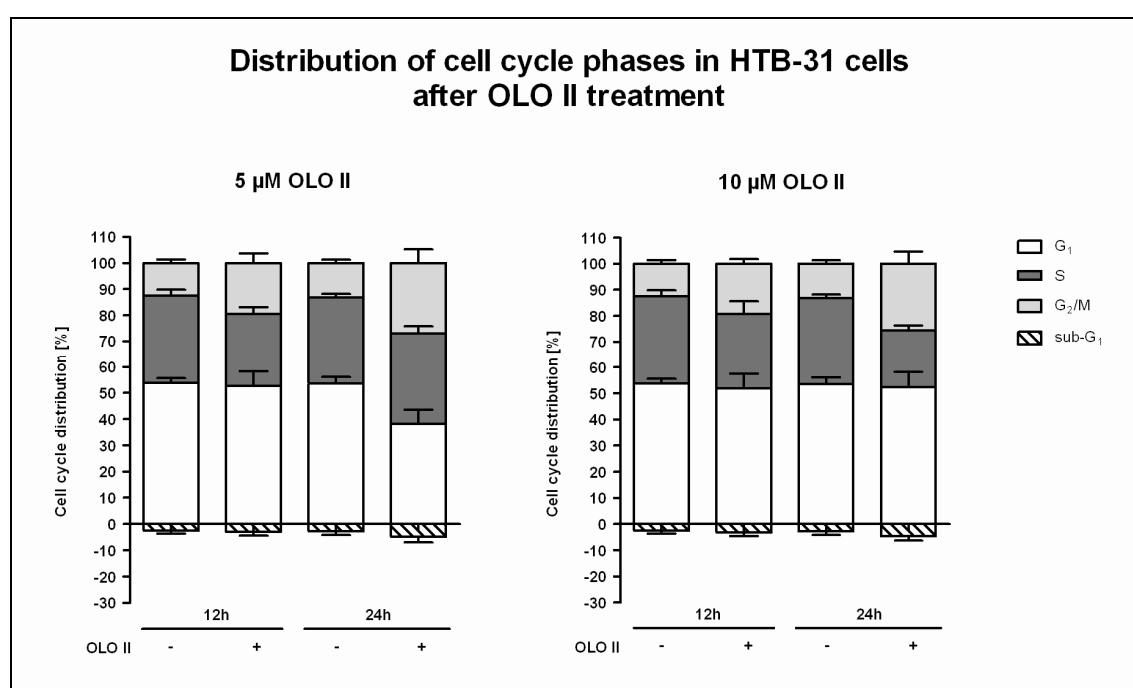


Fig. 45: Distribution of HTB-31 cells in distinct cell cycle phases after OLO II treatment. Treatment of HTB-31 cells with OLO II was performed at the same concentrations and conditions as for HeLa cells.

In summary, all experiments performed so far evidence that inhibition of CDKs by ROSC and OLO II strongly affects the distribution of examined human cervical carcinoma cells in distinct cell cycle phases. Both inhibitors arrested asynchronously growing cells in the G₂/M phase. Moreover, both CDK inhibitors at higher dosage triggered apoptosis. However, unlike HeLa cells, HTB-31 cells were relative resistant to apoptosis and responded to the treatment with a delay.

3.3. Modulation of the major cell cycle regulators upon treatment with selective CDK inhibitors

Anti-proliferative and cell cycle modulatory effects of both examined CDK inhibitors in HeLa and HTB-31 cells evoked the question by which mechanism the drugs induce the observed changes. To address this issue it was necessary to perform detailed analysis of major cell cycle regulators, including cyclin-dependent kinases, by immunoblotting. For this purpose whole cell lysates (WCLs) from control HeLa and HTB-31 cells as well as cells treated with ROSC and OLO II at indicated concentrations were prepared. After protein separation by electrophoresis and subsequent electroblotting onto membranes, distinct proteins including their phosphorylated forms were detected with specific antibodies. Comparison of the expression and optionally phosphorylation status of distinct proteins provided information on their changes upon ROSC/OLO II treatment.

The equal protein loading and proper transfer was always checked by Ponceau S staining. Equal protein loading was additionally verified by incubation of immunoblots with actin, a housekeeping protein that normally does not oscillate in cells upon treatment.

3.3.1. ROSC prevents the activating phosphorylation of CDK2 and CDK1

In the first step, the functional status of the major cell cycle regulators was analyzed. CDK2 and CDK1 are potential targets of both CDK inhibitors and have been inspected first. CDK2 would normally assemble with cyclin E to active complexes and phosphorylate the tumor suppressor pRb to move cells into S phase. CDK1 associates with cyclin B and promotes M phase.

As shown in Fig. 46 ROSC decreased the activating phosphorylation of CDK2 at Thr¹⁶⁰ within the T-loop in a time- and concentration-dependent manner (compared to untreated control cells and to the amount of total CDK2 protein). ^[Paper No. 1] This indicates that ROSC inhibits the activity of CDK2. Already after 12h CDK2 was inactivated by preventing its activating phosphorylation at Thr¹⁶⁰. This was additionally evidenced by further analysis of the modification status of nucleophosmin (NPM), a specific CDK2 substrate, whose phosphorylation at Thr¹⁹⁹ depends on CDK2. Loss of phosphorylation of NPM occurs after treatment for 12h at 40 μ M ROSC and after the further 12h at 20 μ M ROSC. Surprisingly, at this time point the phosphorylated form of NPM appeared (Fig. 47). The relevance of this unexpected effect has to be elucidated.

Consequently, one would expect that inactive CDK2 would be unable to phosphorylate pRb and would stop cell cycle progression in G₁ phase. However, considering the fact that

in HeLa cells the E7 oncoprotein is present, the prevention of pRb phosphorylation could not induce cell cycle arrest at the G₁/S border.

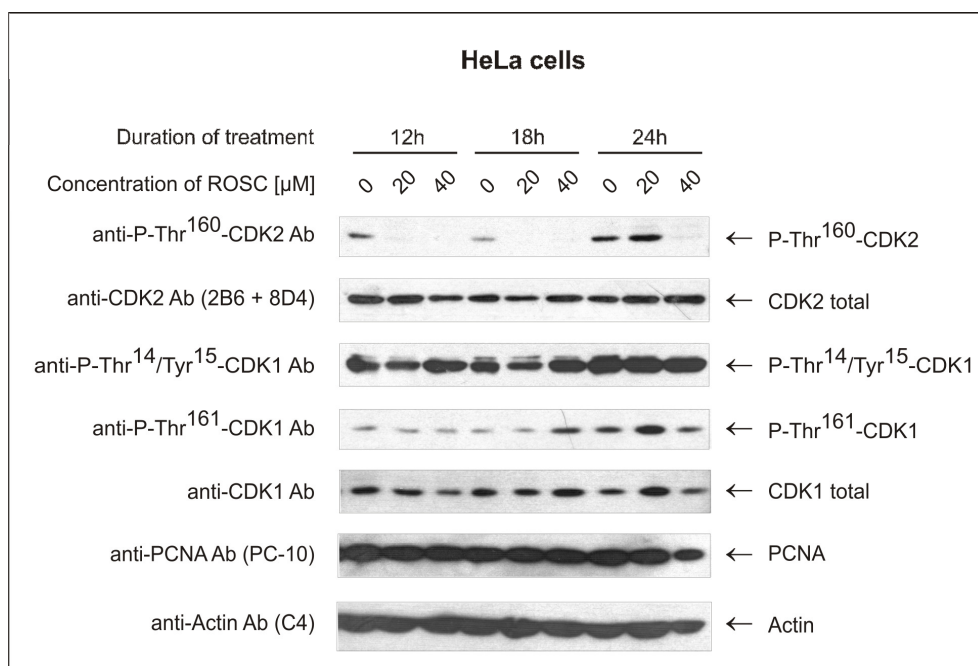


Fig. 46: Abolishing of CDK2 and CDK1 activating phosphorylation after ROSC treatment of HeLa cells. Whole cell lysates from control and ROSC-treated HeLa cells were loaded onto 12 % SDS-PAGE gels, blotted onto PVDF membranes and incubated afterwards with CDK specific antibodies.

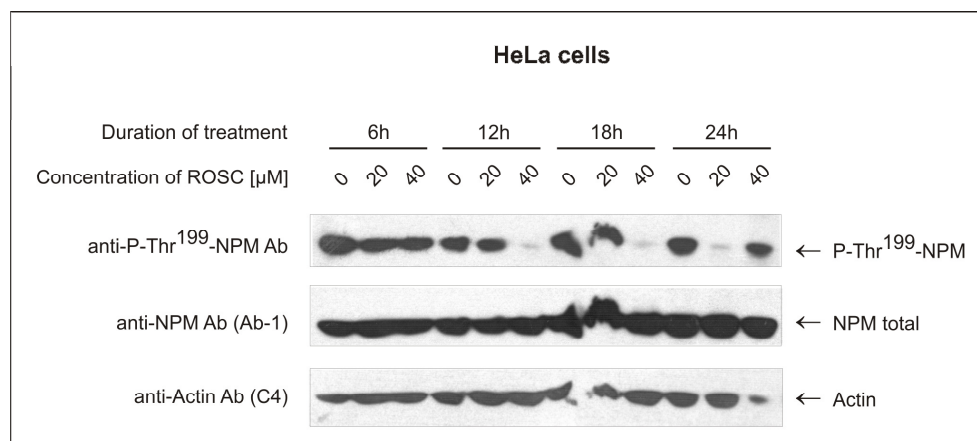


Fig. 47: Decrease of NPM phosphorylation is an evidence for CDK2 inactivation by ROSC. HeLa cells were treated with ROSC and lysed. Proteins were loaded onto 10 % SDS-PAGE gels. Immunoblotting analyses was performed using NPM specific antibodies and anti-actin sera to check the equal protein loading.

Regarding the phosphorylation and activation status of CDK1, obtained results reveal changes of its phosphorylation at three important residues. [Paper No. 1] Phosphorylation at Thr¹⁶¹ prepares CDK1 for its activation, whereas phosphorylation of Thr¹⁴ and Tyr¹⁵ carried out by Wee/Myt1 kinase leads to its inhibition. The obvious increase of the phos-

phorylation of CDK1 at two inhibitory sites beginning after 12h evidenced the inactivation of CDK1 in ROSC-treated HeLa cells. Although the activating phosphorylation of CDK1 slightly increased after 18h as well, it has no importance until no dephosphorylation at inhibitory sites occurs. Inactivation of CDK1 is a main mechanism by which ROSC induces the G₂/M arrest in HeLa cells.

3.3.2. Repression of cdc25C phosphatase correlates with an accumulation of phosphorylation of CDK1 at inhibitory sites

Analysis of the expression and functional status of cdc25C phosphatase that is responsible for dephosphorylation of CDK1 at Thr¹⁴/Tyr¹⁵ revealed that ROSC modulated the phosphorylation status of cdc25C at Ser²¹⁶. The increase of site-specific phosphorylation of cdc25C upon ROSC treatment at a final concentration of 40 μ M for 12h and 18h correlates with the phosphorylation of CDK1 at the inhibitory sites. [Paper No. 1] After treatment for 24h at both ROSC concentrations the amount of phosphorylated and total cdc25C phosphatase significantly decreased (Fig. 48).

Another important player in maintaining the ROSC-induced G₂ arrest is LATS-1, a relative new tumor suppressor protein. LATS-1 was discovered to negatively regulate CDK1, when phosphorylated at Ser⁹⁰⁹, thus inhibiting its kinase function. Thus, ROSC affects CDK1 by three different ways to assure that no cell cycle progress will occur.

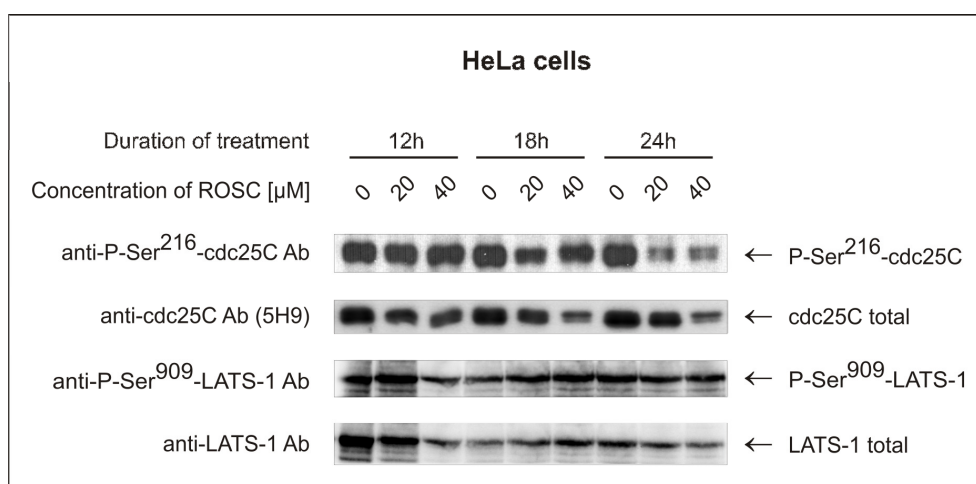


Fig. 48: Cellular levels and phosphorylation status of cdc25C phosphatase and LATS-1 tumor suppressor protein in control and ROSC-treated HeLa cells. HeLa cells were treated with ROSC as indicated and proteins were analyzed by immunoblotting using specific antibodies, as indicated.

3.3.3. ROSC prevents pRb phosphorylation

Loss of CDK2 activation should clearly affect pRb phosphorylation. Therefore, the phosphorylation status of the retinoblastoma protein at three different residues after ROSC treatment was investigated.

ROSC diminished the phosphorylation of pRb protein at Ser⁷⁸⁰ and Ser^{807/811} in a time- and dose-dependent manner, leading to a reactivation of pRb protein functions as a tumor suppressor (Fig. 49).^[Paper No. 1] However, it was unclear whether this event would be able to re-establish the G₁ restriction checkpoint since HeLa cells express HPV-encoded E7 oncoprotein.

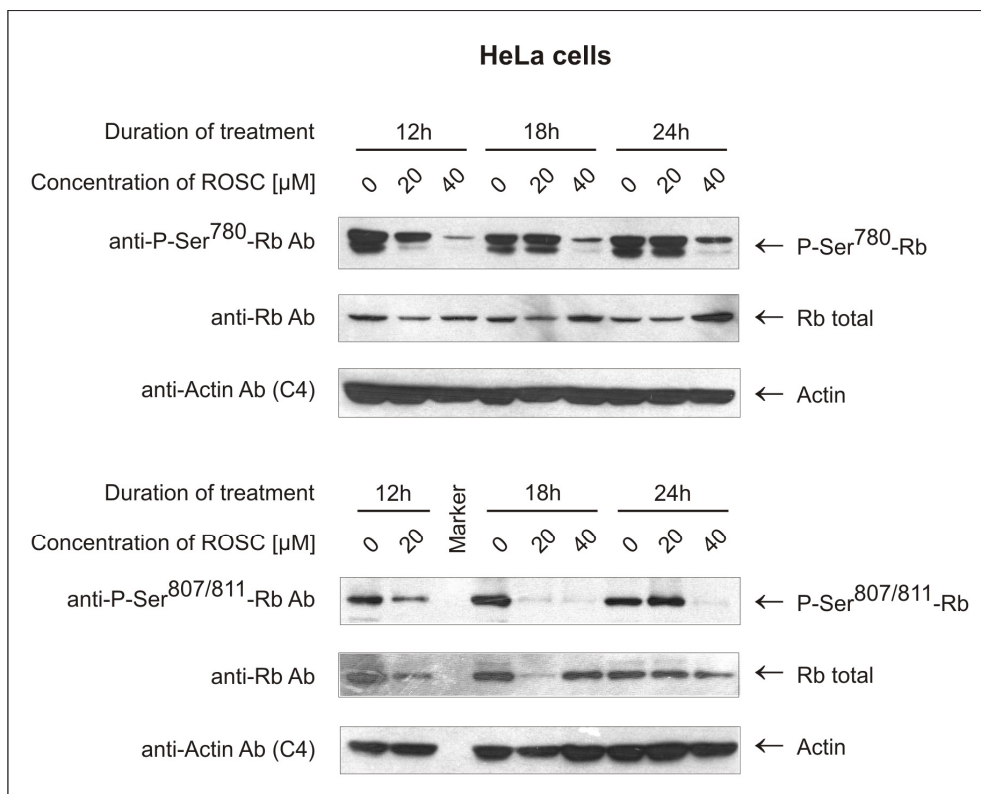


Fig. 49: Reduction of the site-specific pRb phosphorylation. Whole cell lysates obtained from controls and ROSC-treated HeLa cells were loaded onto 10 % SDS-gels and blotted onto PVDF membranes. Afterwards, the protein level and site-specific phosphorylation of pRb was monitored using specific antibodies recognizing pRb phosphorylated at distinct sites as well as total pRb protein irrespective of its modification.

3.3.4. Inactivation of CDK7 mediated by ROSC

Further analyses were performed to determine the current state of CDK7 in untreated control and ROSC-treated HeLa cells. CDK7 is a kinase responsible for the activation of the major cell cycle-related kinases like CDK2 and CDK1 and regulates simultaneously distinct steps in transcription.

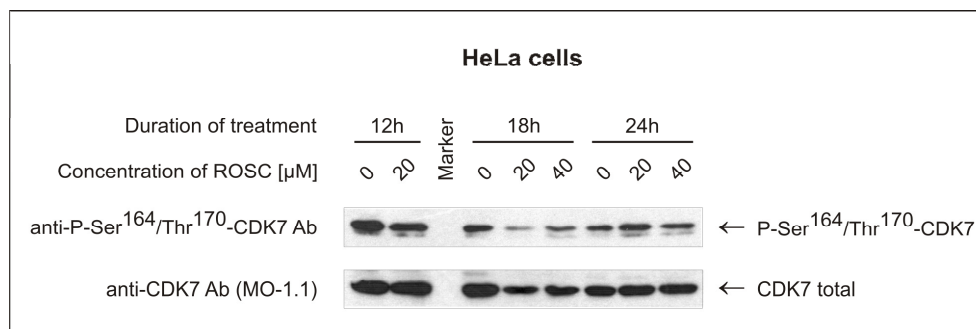


Fig. 50: Abolishing of site-specific phosphorylation of CDK7 in response to ROSC treatment. Site-specific phosphorylation of CDK7 and its total levels were determined in control and ROSC-treated HeLa cells by immunoblotting using specific antibodies.

The functional involvement of CDK7 in the cell cycle and transcription is regulated by a phosphorylation at Ser¹⁶⁴/Thr¹⁷⁰. When HeLa cells were exposed to ROSC, a reduction of the site-specific phosphorylation of CDK7 occurred already after 12h of treatment and decreased to a minimum after the further 6h (Fig. 50). [Paper No. 1 and 2] After a continuous treatment for 24h, phosphorylation of CDK7 at the critical residues slightly increased. However, it seems that a weak increase of phosphorylation is attributable to the elevation of total CDK concentration. These results indicate that reduction of site-specific phosphorylation of CDK7 upon ROSC treatment might correlate with its reduced activity necessary to regulate transcription.

3.3.5. ROSC and OLO II repress the function of RNA polymerase II in HeLa cells necessary for global transcription

One would expect that the prevention of site-specific phosphorylation of CDK7 might strongly affect its functions in the regulation of transcription. CDK7 regulates the activity of RNA polymerase II during transcription. CDK7 catalyzes the phosphorylation of RNA polymerase II at Ser⁵ within the heptapeptid repetitive motif respectively, localized in the carboxy-terminal domain (CTD).

To gain more insight, the functional status of RNA polymerase II was examined in control HeLa cells and cells treated with ROSC as well as OLO II at their most effective concentrations. Whole cell lysate as well as subcellular fraction (isolated nuclei and cytosolic fractions; according to RSB fractionation, section 2.2.13.) were analyzed by immunoblotting. This should facilitate the detection of RNA polymerase II accumulated in nuclei, where transcription occurs.

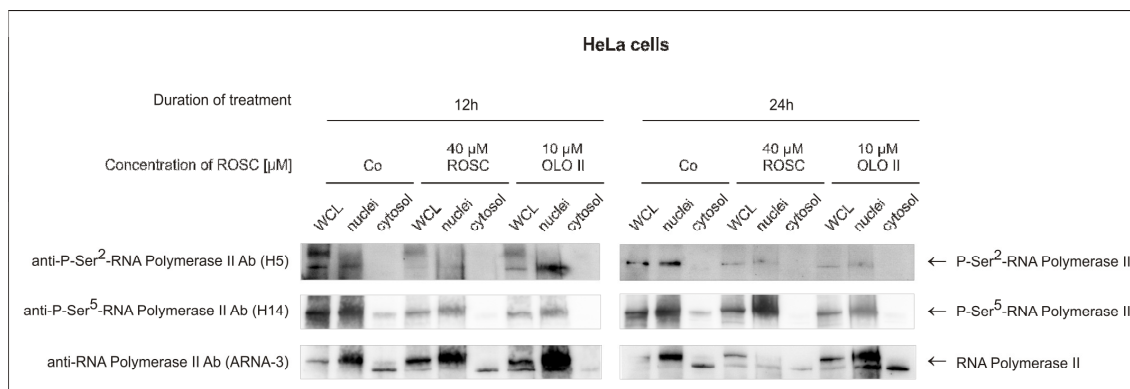


Fig. 51: ROSC and OLO II abolish the phosphorylation of RNA polymerase II at specific serine residues critical for regulation of transcriptional progression. HeLa cells were treated with ROSC and OLO II as indicated. Whole cell lysates and subcellular fractions (nuclei and post-nuclear supernatant representing a crude cytosolic fraction) were loaded onto 8 % SDS-PAGE gels and blotted onto PVDF membranes. Site-specific phosphorylation of the CTD of RNA polymerase II was analyzed by immunoblotting using phosphospecific antibodies from Abcam. Twin blots were used for detection of the phosphorylation of Ser² and Ser⁵. Subsequently, total RNA polymerase II was detected.

The immunoblotting analysis revealed that exposure of HeLa cells to both CDK inhibitors markedly reduced the phosphorylation of RNA polymerase II at Ser⁵ after 12h and this persisted at 24h after action of OLO II (Fig. 51). [Paper No. 2] Interestingly, the phosphorylation of RNA polymerase II at Ser² was completely reduced after 24h (Fig. 51) thereby indicating that the function of RNA polymerase II in the elongation and processing of primary transcripts was inhibited.

3.3.6. Increase of p53 levels upon ROSC treatment

Immunoblotting analysis of whole cell lysates prepared from control and ROSC-treated HeLa cells revealed an increase of p53 protein levels in response to ROSC treatment beginning at 12h. [Paper No. 2] As depicted in Fig. 52, ROSC-mediated enhancement of p53 protein was time and concentration dependent. The highest increase of p53 levels was detected after 24h at a final concentration of 40 μM. In contrast, p53 protein was barely detectable in lysates prepared from untreated control cells. These results indicate that the blockage of global transcription in HeLa cells upon treatment with CDK inhibitors has no effect on the cellular levels of p53 protein. Considering the fact that p53 expression in HeLa cells is primarily controlled by the regulation of protein stability that is determined by HPV-encoded E6 oncoprotein. The upregulation of p53 protein under conditions of the block of global transcription would indicate that both pharmacological CDK inhibitors reduced the expression of HPV-encoded E6 oncoprotein.

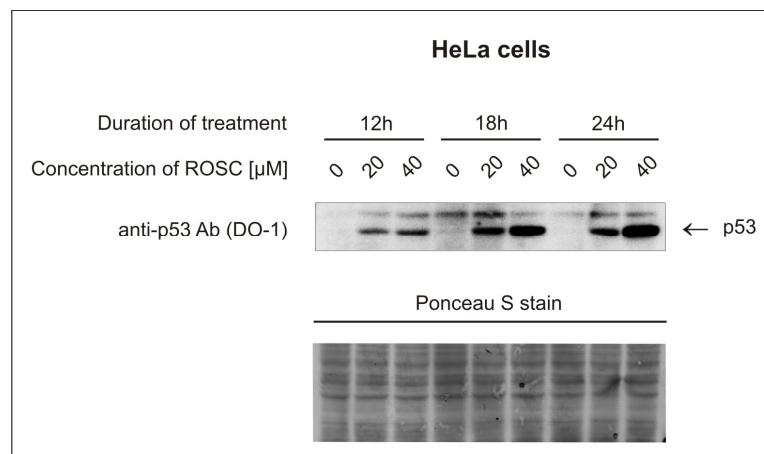


Fig. 52: Increasing levels of p53 protein after ROSC treatment. HeLa cells were treated with ROSC at two different concentrations. Whole cell lysate samples were monitored by immunoblotting for p53 levels. The specific monoclonal DO-1 antibody was used to detect p53.

3.3.7. Repression of E6 and E7 viral oncoproteins in HeLa cells

The viral genome is usually transcribed using tools of host cells and one would expect that blockage of global transcription after inhibition of RNA polymerase II might also affect the expression of the oncogenic proteins E6 and E7, which are integrated into the genome of HeLa cells and co-expressed with other cellular genes. The fact that p53 protein appeared in HeLa cells after treatment with both CDK inhibitors despite blockage of global transcription implicates that the treatment facilitated p53 protein to escape from its E6-mediated degradation. Therefore, it was of importance to prove the effect of both CDK inhibitors on the cellular concentration of HPV-encoded oncoproteins. For this purpose, control and ROSC-treated cells were analyzed for the presence of HPV-encoded proteins. Analysis of whole cell lysate samples revealed the reduction of E7 oncoprotein levels after ROSC treatment for 18h (Fig. 53).^[Paper No. 1] However, the intensity of E7 signal on immunoblots was relatively weak. Therefore, we decided to fractionate cells to enrich the antigens. Since both oncogenic proteins are localized primarily in the nucleus, cells were fractionated yielding the nuclei and post-nuclear supernatant designated as a cytosolic fraction. Then both subcellular fractions (cytosol and nuclei) were analyzed by immunoblotting to detect E6 protein.

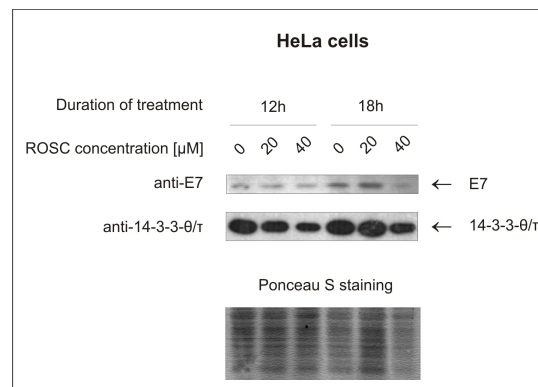


Fig. 53: Repression of E7 oncoprotein by ROSC. HeLa cells were treated with ROSC for 12h and 18h. Whole cell lysates were loaded onto 15 % SDS-PAGE gels. After blotting, E7 was detected using specific anti-E7 antibodies from Abcam.

The repression of HPV 18-encoded E6 protein influenced by ROSC and OLO II treatment is demonstrated in Fig. 54. [Paper No. 2] As expected, E6 oncoprotein was detected in the nuclei isolated from untreated control cells, but not in the cytosolic fraction. The level of E6 oncoprotein was markedly reduced in the nuclei isolated from cells treated with CDK inhibitors. The diminution of E6 protein in whole cell lysates was also observed in samples after treatment for 24h but not at 12h. However, in WCL prepared from untreated control cells at 12h no E6 positive signal was detected. Therefore, it is not possible to evaluate this observation. Both CDK inhibitors repress the E6 protein after 12h and 24h of treatment very effective.

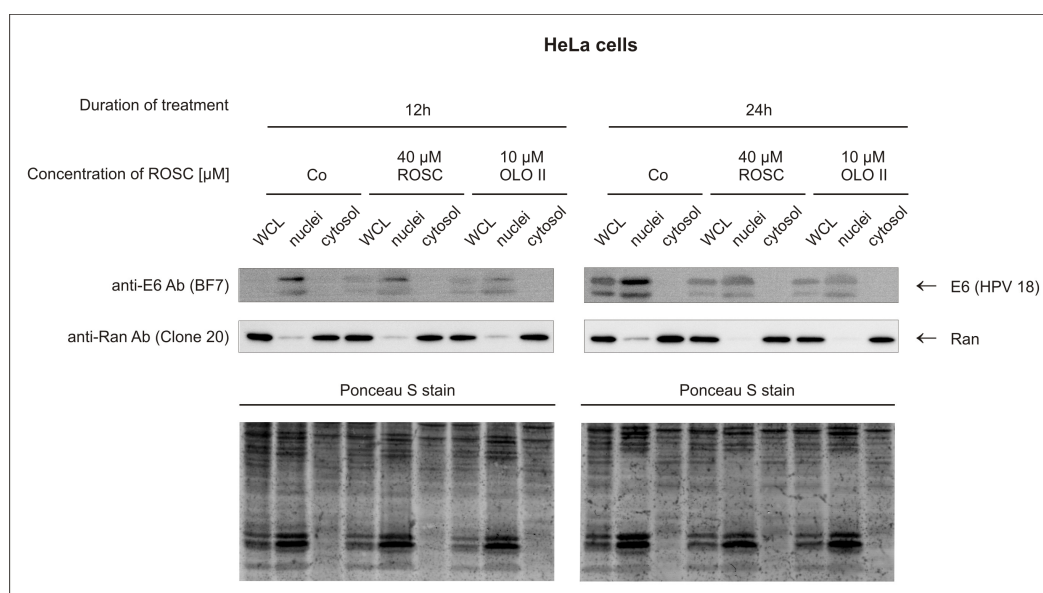


Fig. 54: Repression of E6 oncoprotein expression by ROSC and OLO II. Controls and ROSC and OLO II-treated HeLa cells were lysed yielding whole cell lysates or fractionated nuclei. Proteins were separated onto 15 % SDS-PAGE gels and blotted onto PVDF membranes. E6 specific antibodies from Abcam allowed the detection of the oncoprotein. To prove the authenticity of the cytosolic fraction and the equal protein loading detection of Ran, a small GTPase, occurred. Ran was only detected to low levels in nuclei, a fact which may be influenced by its ability of shuttling between cytosol and nuclei at any time.

We conclude that ROSC and OLO II clearly display anti-viral activity. They are definitely able to inactivate the oncogenic E6 and E7 proteins in HeLa cells by modulating the RNA polymerase II functional status and in consequence the global transcription.

3.3.8. Site-specific phosphorylation of CDK7, but not that of CDK2 is affected in HTB-31 cells

Unlike HeLa cells, HTB-31 cells were less sensitive to the action of CDK inhibitors. Therefore it was unclear, which cell cycle regulators might be affected by ROSC and OLO II.

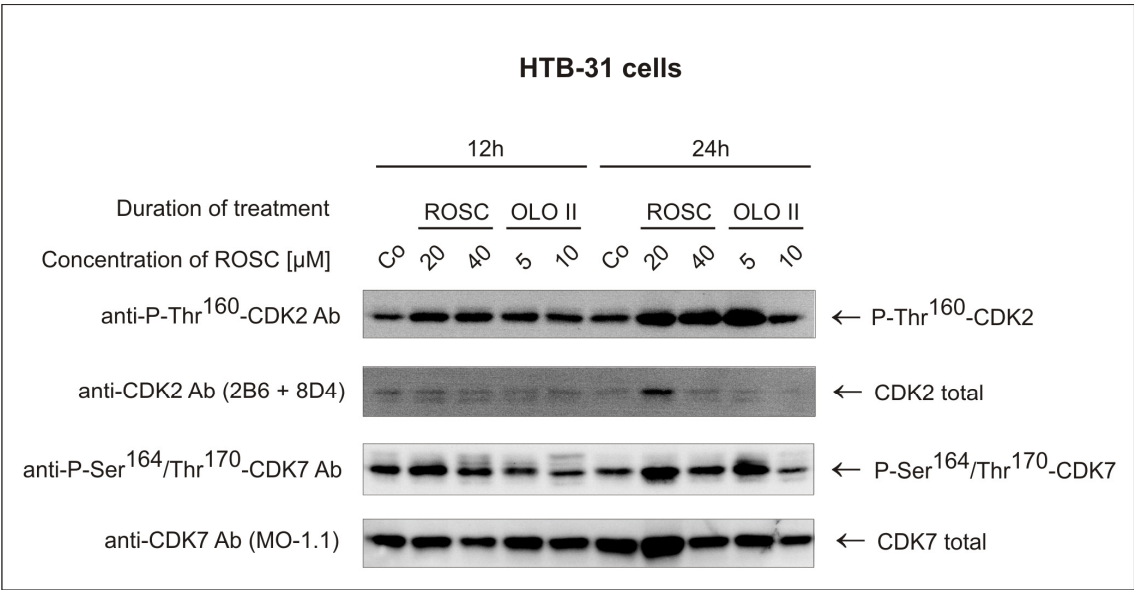


Fig. 55: Effect of ROSC and OLO II on the functional status of CDK7 and CDK2 in human HTB-31 cells. HTB-31 cells were treated with ROSC and OLO II at two different concentrations for 12h and 24h. Whole cell lysates were generated and analyzed for the presence of an activating phosphorylation in CDK2 and CDK7. The sample obtained from the treatment with 20 μM ROSC for 24h was overloaded and for this reason it has not been considered at all. The reactivity of endogenous CDK2 in HTB-31 cells with monoclonal anti-CDK2 antibodies was reduced as compared to that in other human cancer cells.

Immunoblotting experiments revealed that both tested CDK inhibitors did not reduce the site-specific phosphorylation of CDK2 at Thr¹⁶⁰ (Fig. 55). Surprisingly, after exposure of HTB-31 cells to ROSC the phosphorylation of CDK2 at Thr¹⁶⁰ increased. This unexpected effect became evident especially after treatment for 24h.

In contrast, both CDK inhibitors were able to reduce the activating phosphorylation of CDK7. Decrease of the phosphorylation of CDK7 occurred only at higher ROSC concentration (Fig. 55). These results show that the functional status of both examined kinases was differentially modulated by tested CDK inhibitors.

3.3.9. Transcriptional regulation in HTB-31 cells is affected by OLO II

Decrease of site-specific phosphorylation of CDK7 in response to CDK inhibitors indicates that they might interfere with cellular transcription. Therefore, the functional status of CTD of RNA polymerase II was examined in HTB-31 cells exposed to CDK inhibitors.

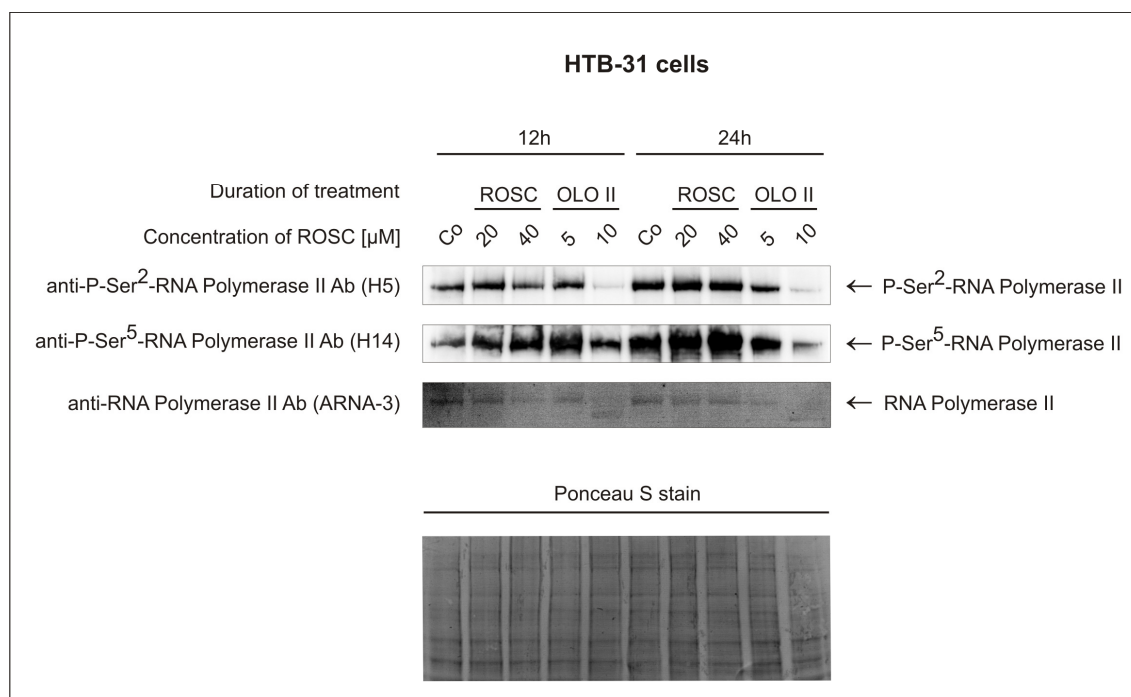


Fig. 56: Repression of cellular transcription in HTB-31 cells by OLO II. HTB-31 cells were treated with ROSC and OLO II at the indicated concentrations for 12h and 24h. Whole cell lysates were analyzed for the phosphorylation of RNA polymerase II using specific antibodies. However, there were some problems with the detection of the total RNA polymerase protein.

As expected, decrease of the phosphorylation of CDK7 at sites promoting its affinity to CTD template also affected the phosphorylation of RNA polymerase II at Ser² and Ser⁵ in HTB-31 cells. However, only OLO II was able to strongly reduce the modification of RNA polymerase II (Fig. 56). Abrogation of the phosphorylation at Ser² was induced by OLO II at a concentration of 10 μM already after 12h and maintained for further 12h. 5 μM OLO II was able to diminish the same phosphorylation only after 24h of treatment. This indicates that lower OLO II concentrations need a longer time to exert their functions on HTB-31 cells. Regarding the phosphorylation at Ser⁵, only the highest OLO II concentration reduced it significantly.

ROSC seems to have a low, if any, effect on RNA polymerase II in HTB-31 cells. ROSC at a final concentration of 40 μM decreased the phosphorylation at Ser² after 12h very well, but this effect was transient. These results show that both CDK inhibitors differentially affect their major cellular targets in HTB-31 cells.

3.3.10. Neither ROSC nor OLO II affects p53 levels in HTB-31 cells

In further tests the effect of medication on p53 levels in HTB-31 cells was monitored. These cells harbour a p53 mutation which causes the accumulation of the malfunctioned protein, which is then unable to induce cell cycle arrest.

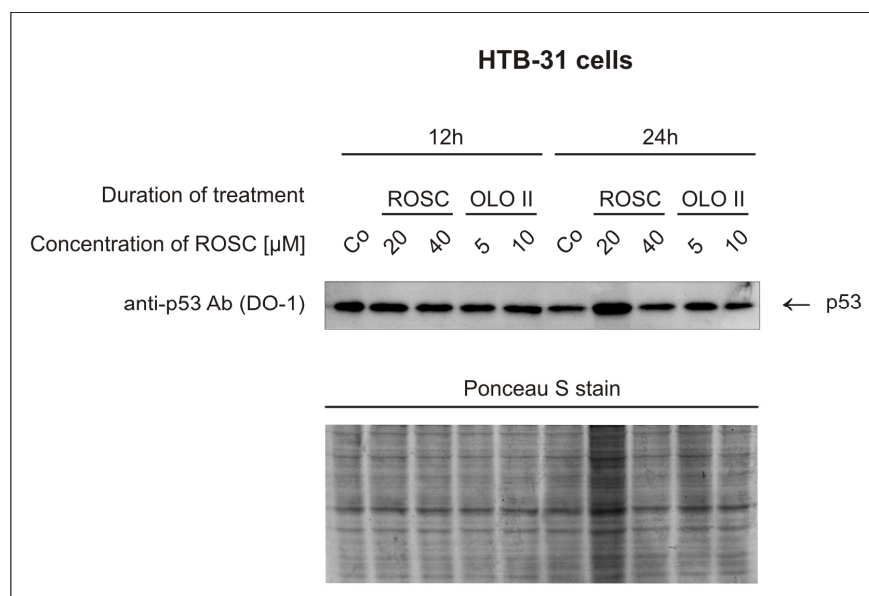


Fig. 57: ROSC and OLO II treatment does not affect levels of p53 protein in HTB-31 cells. HTB-31 cells were treated with ROSC and OLO II at two different concentrations. Whole cell lysates were monitored for p53 levels by immunoblotting. The specific monoclonal DO-1 antibody was used to detect p53. The sample obtained after treatment with 20 μM ROSC for 24h was overloaded due to an underestimation of protein concentration and was not taken into consideration.

As shown in Fig. 57, treatment of HTB-31 cells with ROSC and OLO II did not affect p53 levels at all. Intensity of p53 protein band remained nearly constant throughout the treatment period. This result indicates that mutated p53 expressed in HTB-31 cells has prolonged stability and remains unaffected by treatment with tested CDK inhibitors.

3.3.11. ROSC induced upregulation of NF-κB in HTB-31 cells

NF-κB is a transcription factor usually induced upon environmental stress (e.g. infection, inflammation etc.) capable of activating downstream genes to reprogram gene expression. Besides its functions in mounting the innate immune response, it is known to be involved in promoting survival that contributes to carcinogenesis as well. However, the biological function of NF-κB seems to depend on the cellular context. Thus, in some cases repression of NF-κB prevents tumor formation. ^[171]

As shown in Fig. 58 the phosphorylation status of NF-κB protein changed during cultivation of control HTB-31 cells. At 12h after the onset of the experiment Ser⁵³⁶ was unmodified and after further 12h a strong phosphorylation of NF-κB at Ser⁵³⁶ was detected. Inter-

estingly, exposure of HTB-31 cells to ROSC for 12h and 18h strongly enhanced the site-specific phosphorylation of the NF- κ B protein. However, at 24h the intensity of NF- κ B protein phosphorylated at Ser⁵³⁶ in control and ROSC-treated cells was comparable. Unlike the phosphorylated form, the total level of NF- κ B was not affected by ROSC. These results indicate that ROSC-mediated enhancement of site-specific phosphorylation of NF- κ B protein observed at 12h and 18h modulates its activity and not its steady-state. This event might be linked to its pro-apoptotic functions involving FAS/FAS ligand-mediated pathway.

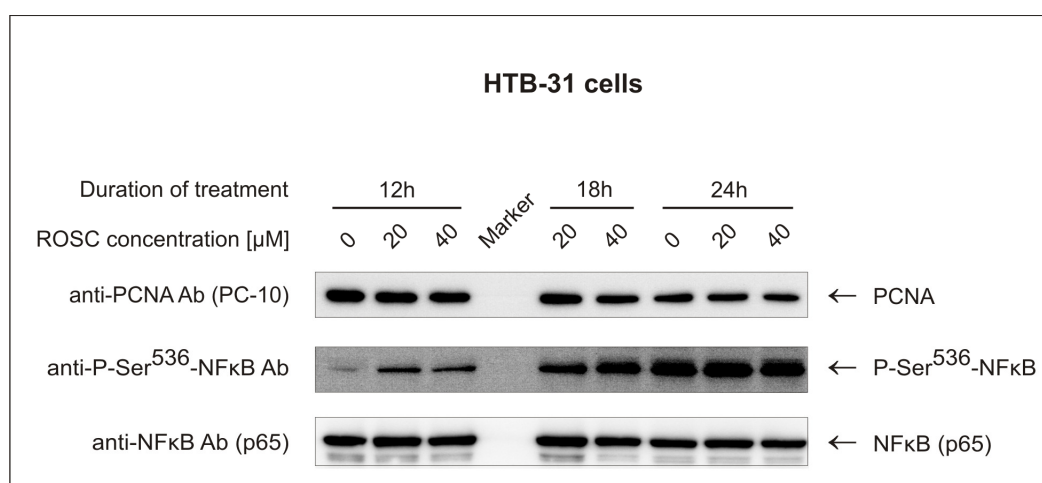


Fig. 58: ROSC enhanced site-specific phosphorylation of NF- κ B in human HTB-31 cells in a time- and concentration-dependent manner. HTB-31 cells were treated with ROSC and analyzed for the activity of NF- κ B protein after 12h, 18h and 24h.

The systemic analysis of the changes of the major cell cycle regulators in HeLa and HTB-31 cells after treatment provided more insight into the mode of action of the pharmacological inhibitors ROSC and OLO II. In comparison to HeLa cells, HTB-31 cells are less sensitive to these inhibitors.

3.4. Apoptosis-promoting effects induced by the CDK inhibitors ROSC and OLO II in HeLa and HTB-31 cells

DNA profiles (section 3.3.), showing the changes in the cell cycle progression after treatment, clearly show the appearance of a hypoploid cell population after medication, which seems to represent cells undergoing apoptosis. To prove the assumption, distinct assays allowing to detect cells undergoing apoptosis were performed in HeLa and HTB-31 cells. Moreover, it was of interest to determine by which mechanisms CDK inhibitors would be able to induce apoptosis.

3.4.1. Detection of caspase-3-cleaved cytokeratin 18 in cancer cells exposed to CDK inhibitors

Cleavage of cytokeratin 18, a component of cytoskeleton, mediated by activated effector caspase-3 is a characteristic feature of epithelial cells undergoing apoptosis. HeLa cells were treated with ROSC and OLO II at concentrations used before. After fixation the samples were stained with the FITC-coupled monoclonal M30 CytoDEATH antibody recognizing solely caspase-3-cleaved cytokeratin 18. This technique provides an easy and fast way to detect ongoing apoptosis *in situ*. Normal healthy cells remain unstained. However, in cells with activated caspase cascade a characteristic punctuated staining pattern appears in the cytoplasm, an indication for the presence of the truncated form of cytokeratin 18. Compared to controls, which simply display a weak diffuse green fluorescent background, treated HeLa cells definitely show a strong green speckled fluorescent staining pattern (Fig. 59). Counterstaining with DAPI evidenced that not all cells in the field were reactive with M30 CytoDEATH antibody. Generally, the higher ROSC and OLO II concentrations seem to induce cell death at a higher rate than the lower dose. In contrast, in response to DOX treatment, the majority of cells exhibited positive reaction with M30 CytoDEATH indicating that DOX induced massive cell death. Unlike in HeLa cells, DOX had a very weak effect on HTB-31 cells. The results revealed by M30 CytoDEATH staining correlate with DNA histograms and confirm previous assumption that inhibition of cellular CDKs by pharmacological antagonists induced apoptosis in HeLa cells but not in HTB-31 cells.

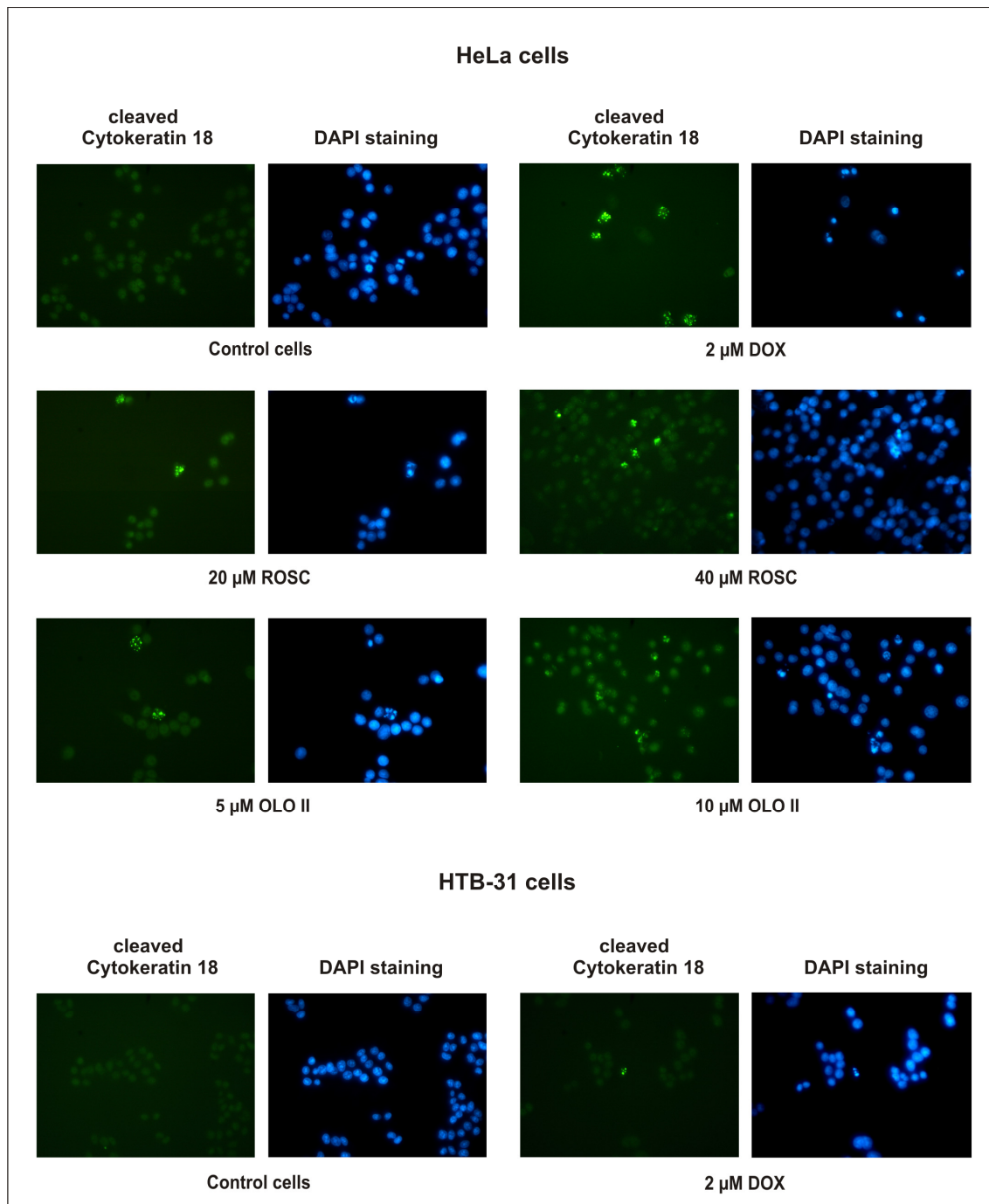


Fig. 59: Detection of caspase-3-cleaved cytokeratin 18 in human cervix carcinoma cells exposed to drugs. Control and drug-treated HeLa and HTB-31 cells were fixed with MeOH and permeabilized using Triton-X 100. Thereafter specimens were overnight incubated with M30 CytoDEATH antibody. Chromatin was visualized after staining with DAPI. Pictures were taken at the 40x magnification objective.

3.4.2. Loss of J-aggregate formation upon treatment of HeLa and HTB-31 cells indicate the collapse of the mitochondrial membrane

In the further experiments the effect of drugs on the potential of mitochondrial membrane was examined. Control and treated cells were stained with the fluorescent dye JC-1. The reaction was evaluated in two independent approaches. First, the aggregation of the dye was assessed *in situ* by immunofluorescence microscopy. Secondly, the emitted fluorescence was analyzed in two channels by flow cytometry. JC-1 provides information on the integrity of mitochondrial membrane potential. Using JC-1 dye for staining, the collapse of the mitochondrial membrane, an early event in apoptosis, can be detected and quantified. Normally, JC-1 forms J-aggregates generating punctuated red fluorescence accumulating at the charged mitochondrial membrane in intact cells. Apoptotic cells, however, display a reduced membrane potential, where JC-1 fails to form dimers and remains monomeric, thus emitting a green fluorescence.

In situ staining of HeLa cells was performed after a 24h treatment with OLO II or DOX using 20 μ M JC-1 (see section 2.2.11.). Immunofluorescence images are depicted in Fig. 60. Cells were first visualized by the Hoffman modulation contrast and then the generated fluorescence was analyzed. Cells displaying the normal potential of mitochondrial membrane are stained red, whereas apoptotic cells appear to be green. Apparently, the red fluorescence is quite strong and shines in the green light channel as well. This facilitates the discrimination between intact and apoptotic cells. In comparison to controls, treated cells clearly display a higher frequency of apoptotic cells, that are green stained due to dissipation of the potential of the mitochondrial membrane.

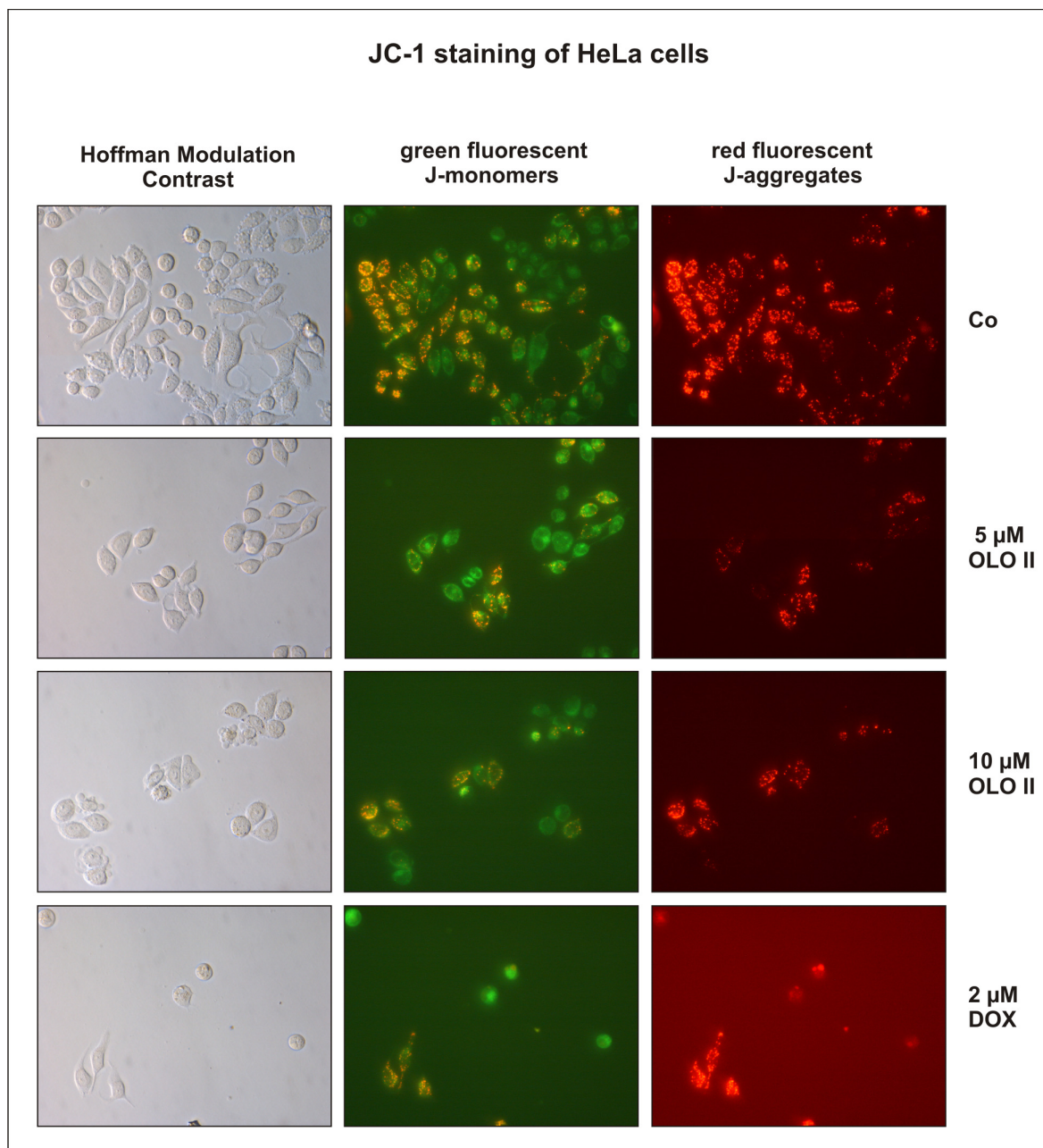


Fig. 60: Accumulation of monomeric JC-1 dye upon collapse of mitochondrial membrane after OLO II and DOX treatment. HeLa cells were treated with OLO II and DOX at different concentrations for 24h and stained with 20 μM JC-1. By changing the excitation light, green and red fluorescence were recorded. Pictures were taken at the 40x magnification objective. DOX is a DNA-intercalating dye, which upon intercalation and excitation emits red fluorescence. Therefore, red fluorescence in DOX-treated HeLa cell images represents its own fluorescent properties, since J-aggregates are barely accumulated. However, DOX fluorescence was not independently determined.

The staining procedure for determination of the potential of the mitochondrial membrane by flow cytometry is very similar to that used for evaluation under immunofluorescence microscopy. HeLa and HTB-31 cells were simply incubated with JC-1 at a final concentration of 20 μM for 20 minutes, carefully washed and afterwards immediately measured to avoid bleaching over time. Flow cytometric measurement of JC-1-stained cells allows

quantification of the rate of apoptosis mediated via the mitochondrial pathway. Usually at least 20,000 cells were measured.

In HeLa cells DOX at a concentration of 2 μM clearly disturbed the membrane potential in 50 % of all stained cells leading to the collapse of the mitochondrial membrane (Fig. 61). Thus, DOX triggered apoptosis very efficiently already after 24h. After continuous treatment for 48h even the tenfold lower DOX concentration induced apoptosis at a high rate. The apoptosis was triggered with similar frequency also after a discontinuous treatment modus (Fig. 61, diagram in the middle).

In contrast, ROSC and OLO II are less potent than DOX. However, they are also capable to induce apoptosis via mitochondrial pathway at higher doses. ROSC at a final concentration of 40 μM induced apoptosis up to 15 % within 24h. This ratio slightly increased after longer incubation periods. The pro-apoptotic effect of OLO II was weaker than that of ROSC.

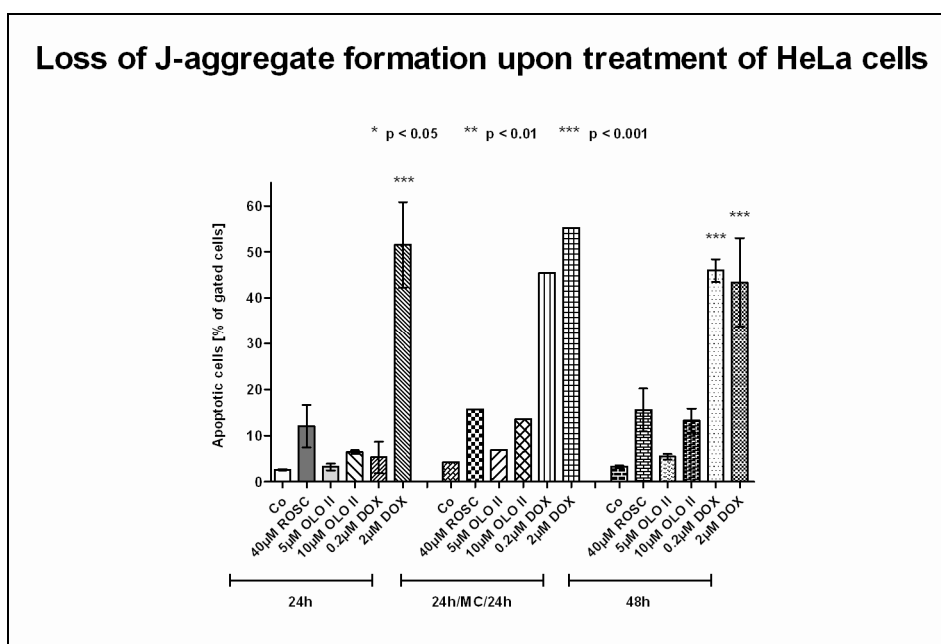


Fig. 61: Loss of J-aggregate formation upon treatment of HeLa cells. HeLa cells were treated with ROSC, OLO II and DOX for the indicated periods of time and then stained with JC-1. Emitted fluorescence was measured in two channels. Quantification analyses of apoptotic cells were performed by flow cytometry. No statistical analyses have been done for results after discontinuous treatment (24h/MC/24h).

Considering that fact that after treatment of HTB-31 cells with DOX, a strongly cytotoxic agent, for 24h a sub- G_1 population was barely detectable, the duration of the treatment scheme was slightly modified. The treatment was constricted to extended time periods. Flow cytometric results shown in Fig. 62 confirm once again the higher resistance of HTB-31 cells to distinct drugs. DOX at the highest concentration induced apoptosis after 48h in

approximately 26 % of cells. At this time point only OLO II exerted comparable effects. Thus, unlike in HeLa cells, in HTB-31 cells OLO II was generally more effective than ROSC.

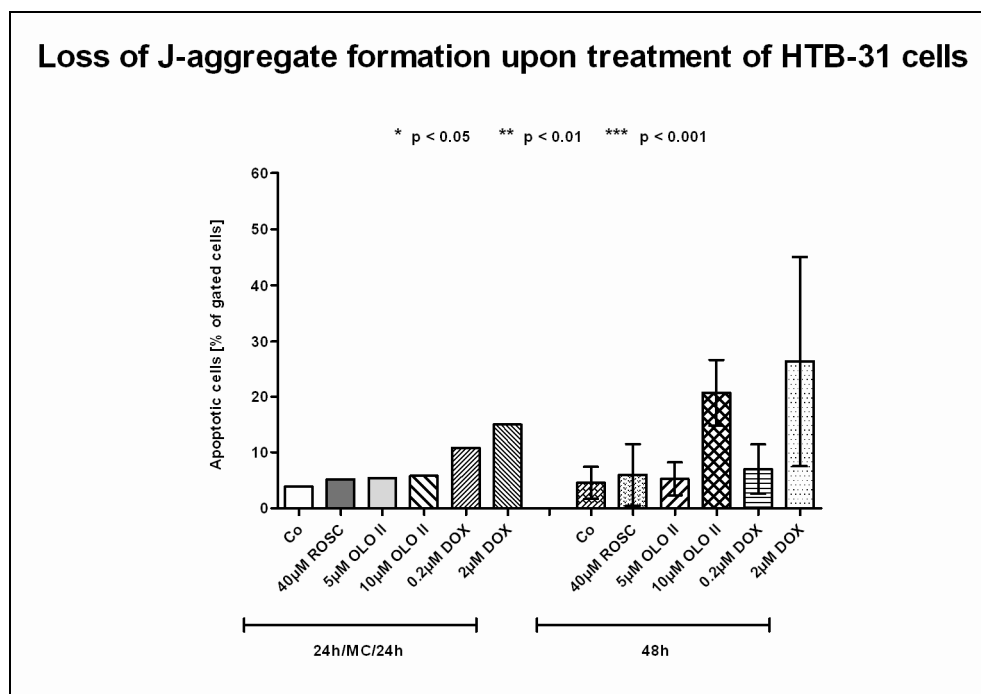


Fig. 62: Loss of J-aggregate formation upon treatment of HTB-31 cells. HTB-31 cells were stained with JC-1 at a final concentration of 20 µM. Emitted fluorescence was measured in two channels. Quantification analyses of apoptotic cells were performed by flow cytometry. No statistical analyses have been done for results after discontinuous treatment (24h/MC/24h).

Taken together, the above described experiments revealed a weak to moderate effect of both examined CDK inhibitors on the potential of the mitochondrial membrane of HTB-31 and HeLa cells, respectively. The very strong effect of DOX on the potential of the mitochondrial membrane in HeLa cells reflects very well its highly cytotoxicity and evidences that the method satisfyingly worked.

3.4.3. Intracellular accumulation of 7-AAD as an evidence for the loss of the plasma membrane integrity

Previously performed assays revealed that ROSC and OLO II are able to induce apoptosis in HeLa and in a lower rate in HTB-31 cells. In the next experiments we addressed the question whether both examined CDK inhibitors are cytotoxic. For this purpose the dye exclusion test was performed. Very potent drugs that damage DNA and generate free radicals are highly cytotoxic resulting in necrosis and not apoptosis. A typical property of necrosis is the loss of the plasma membrane integrity. In contrast, cells in early stages of

apoptosis still display an intact membrane. The status of the cell membrane may be checked by the simple incubation of cells with dyes that are excluded by those with the intact plasma membrane. Dyes like 7-AAD, after entering cells, accumulate in the nucleus and intercalate into DNA allowing the quantification of cells with a leaky plasma membrane by flow cytometry (procedure see 2.2.8.).

HeLa and HTB-31 cells were both tested for the necrotic properties of certain drugs.

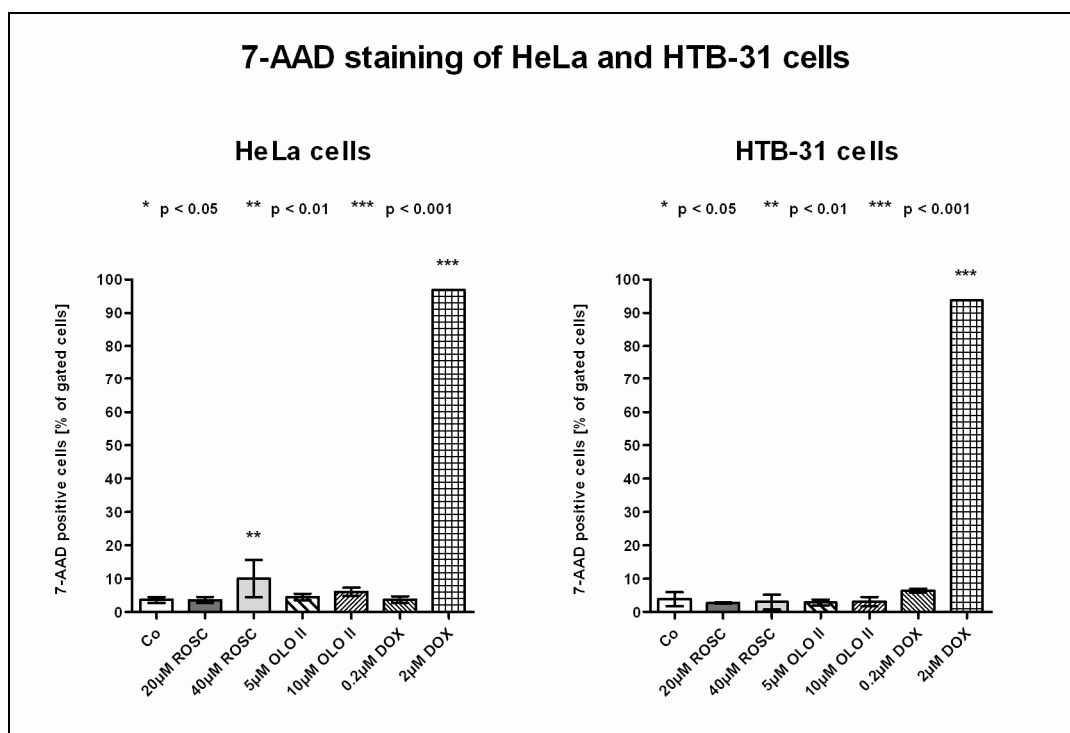


Fig. 63: Uptake of 7-AAD is an evidence for the loss of the membrane integrity. HeLa and HTB-31 cells were treated with the indicated drugs for 24h and thereafter stained with 7-AAD for 20 minutes. Samples were measured by flow cytometry.

The dye exclusion test revealed a high cytotoxicity of DOX at a higher concentration, whereas both examined CDK inhibitors even at high concentrations impaired the plasma membrane only in a low number of cells (Fig. 63).

Considering the fact that the rate of ROSC and OLO II induced apoptosis exceeds that of 7-AAD accumulation, it seems that they did not generate necrosis in HeLa and HTB-31 cells.

3.4.4. ROSC and OLO II initiate caspase-dependent apoptosis in HeLa cells

So far performed cell-based assays showed that CDK inhibitors induce a programmed cell death in HeLa cells. The detection of cleavage of cytokeratin 18 in HeLa samples treated with both CDK inhibitors pointed out that they induce caspase-dependent apoptosis. A

second indication was provided by the fact that CDK inhibitors induced apoptosis by the mitochondrial pathway. Dissipation of the potential of the mitochondrial membrane resulting in a release of cytochrome c is known to trigger the activation of caspase-cascades.

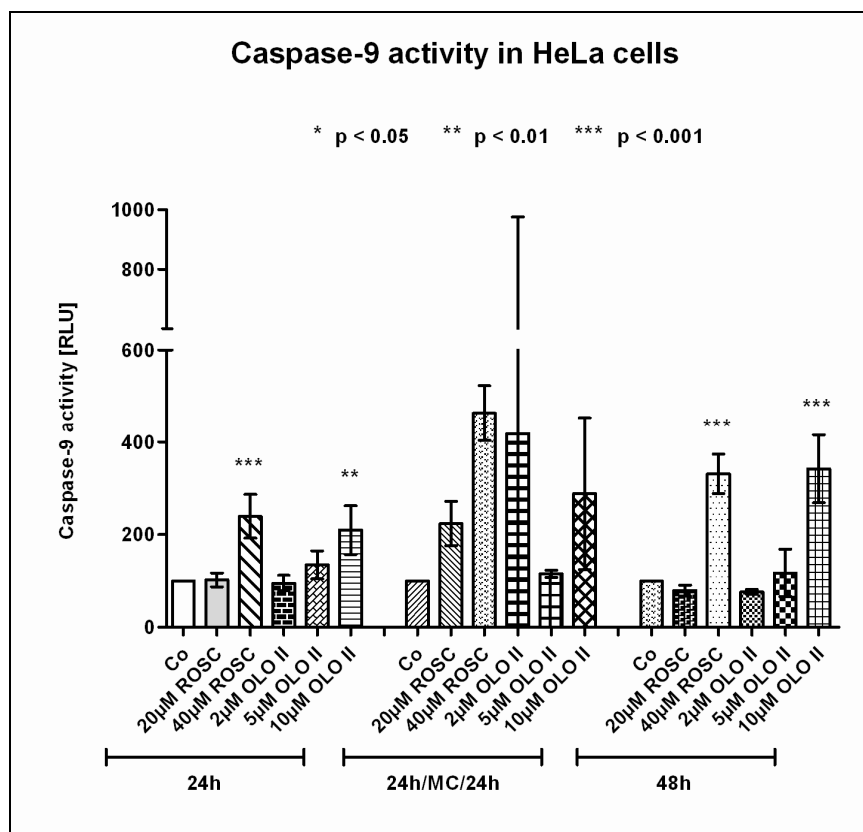


Fig. 64: Activation of initiator caspase-9 in control and drug-treated HeLa cells. HeLa cells were treated with different ROSC and OLO II concentrations for different time points. The activity of caspase-9 was determined using the Caspase-Glo® 9 assay from Promega. The caspase-9 activity was determined as relative luminescence units (RLU) and normalized to the number of viable cells assessed by proliferation assays as earlier described. Results obtained for 2 µM OLO II after a 24h/MC/24h treatment seemed not to match with the other results, thus they were not taken into consideration.

Determination of enzymatic activity revealed that ROSC and OLO II induce caspase-9 activity in a concentration- and time-dependent manner. Both drugs activated caspase-9 at higher doses (Fig. 64). Interestingly, the strongest activation of caspase-9 was not observed after a long continuous but after a discontinuous treatment modus (treatment for 24h with a subsequent medium change and a post-incubation for 24h in a drug-free medium). However, the continuous treatment for 48h was of advantage only in the case of OLO II. As expected, DOX strongly activated the initiator caspase-9 in HeLa cells already after short treatment (data not shown).

To substantiate the obtained results, the activity of downstream effector caspase-3 and -7 was performed using two different assays. This should offer the possibility to obtain method-independent results. Additionally, caspase-3/7 activity was measured in cells as

well as in their culture medium (supernatant) to find out at which time point plasma membrane becomes permeable resulting in leakage of activated caspases into the culture medium.

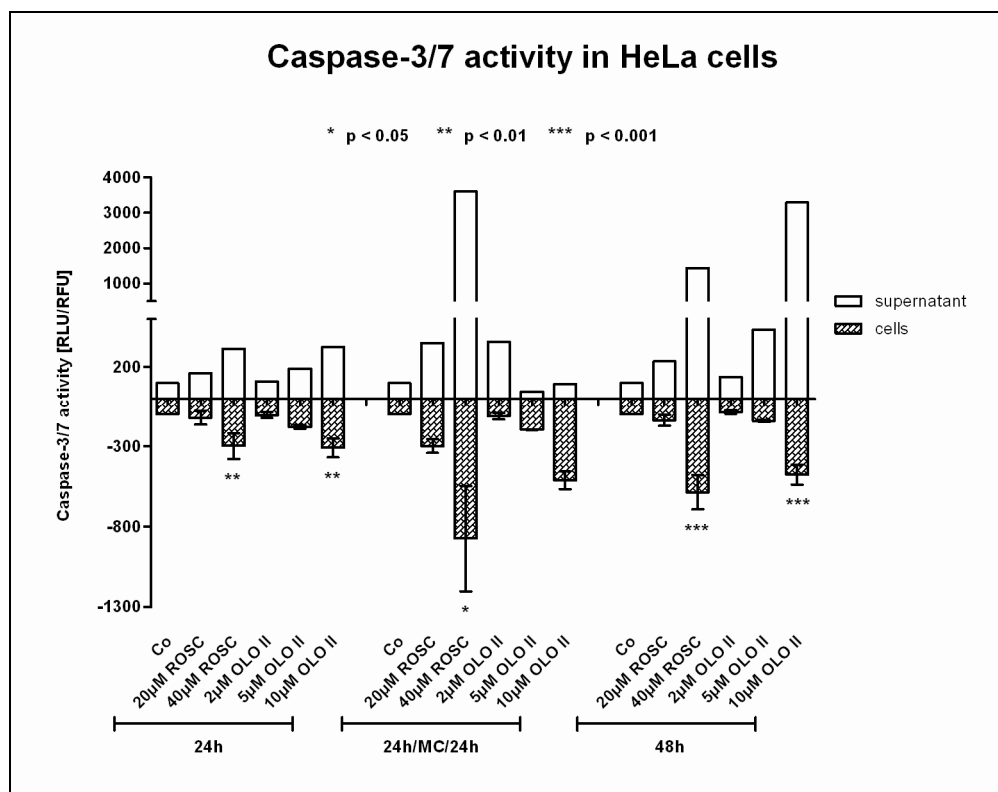


Fig. 65: Enhancement of caspase-3/7 activity after treatment of HeLa cells with CDK inhibitors. ROSC and OLO II treatment of HeLa cells was performed as indicated. Caspase-3/7 activity was measured in treated cells using Promega's Caspase-Glo® 3/7, and independently in culture medium using Apo-ONE® assay. The caspase-3/7 activity was stated as relative luminescence units (RLU) or alternatively as relative fluorescence units (RFU) and normalized to the number of viable cells assessed by CellTiter-Glo® luminescent cell viability assay.

Fig. 65 demonstrates the strong correlation of results regarding the activation of caspase-9 and caspase-3/7. In samples with elevated caspase-9 activity, induction of caspase-3/7 activity was also detected. Likewise, caspase-3/7 accumulation is strongest after a 24h treatment with a subsequent medium change and a post-incubation in a drug-free medium. This applies to both CDK inhibitors. Thus, ROSC and OLO II show their potency towards caspase-3/7 induction, confirming ROSC to be the more potent inducer.

After a longer treatment period (48h) the release of effector caspases into the culture medium was observed (results obtained for supernatants from 40 µM ROSC after a 24h/MC/24h treatment were not taken into consideration, since they appear to be strange). Caspase-3/7 accumulation was threefold higher in supernatants than in ROSC-treated cells

and nearly sevenfold higher in OLO II supernatants compared to OLO II-treated cells. The other treatments barely discharged any caspases to the medium.

In conclusion, ROSC and OLO II induce caspase-dependent apoptosis by an approximately fourfold activation of initiator and effector caspases over the levels observed in control cells already after 24h of treatment. The long-term effects of CDK inhibitors became evident, when caspases were released into the culture medium.

3.4.5. Phosphorylation of Bad and survivin contributes to ROSC-induced apoptosis

Further experiments were performed to investigate by which mechanisms ROSC induces apoptosis. Control and ROSC-treated HeLa cells were therefore lysed and the expression as well as the functional status of selected cell death regulators, amongst others Bad and survivin, were analyzed.

The first two proteins detected were pro-caspase-3 and poly [ADP-ribose] polymerase-1 (PARP-1), its nuclear downstream substrate. Pro-caspase-3 represents the inactive pro-zymogen of caspase-3, which in turn of apoptotic signals becomes proteolytically cleaved. ROSC at a concentration of 40 μ M clearly reduced the levels of inactive pro-caspase-3 after 18h and 24h of treatment thereby indicating its cleavage and following activation (Fig. 66). These results are in concordance with those delivered by caspase assays. Likewise, analysis of PARP-1 confirmed the activation of effector caspases, since levels of full-length PARP-1 protein decreased after 24h. However, it is quite surprising that no other cleavage product of PARP-1 could be detected. The monoclonal C-210 anti-PARP-1 antibody recognizes an epitope at the NH₂-terminal part of the 85 kDa cleaved fragment. Additionally, it is puzzling why PARP-1 is not depleted after 18h of treatment.

Further investigations revealed a decrease of the inhibitory phosphorylation of the Bad protein at Ser¹¹² after 12h. [Paper No. 1] Its phosphorylation was completely abolished after further 12h of treatment. Thus, ROSC obviously targets Bad for reactivation to heterodimerize with Bcl-2 pro-survival proteins. This inhibits consequently their function and promotes cell death by mitochondrial breakdown. Concomitantly, the amount of 14-3-3- θ/τ protein, the sequestering partner of phosphorylated Bad, decreased already after 12h. Both proteins (Bad and 14-3-3- θ/τ) show these effects, when exposed to 40 μ M ROSC.

Lastly, survivin, an inhibitor of apoptosis, was examined. ROSC at a higher dose reduced its phosphorylation as well, an effect observed after 18h of treatment. Since this site-

specific modification modulates survivin's stability, its protection from apoptosis was compromised.

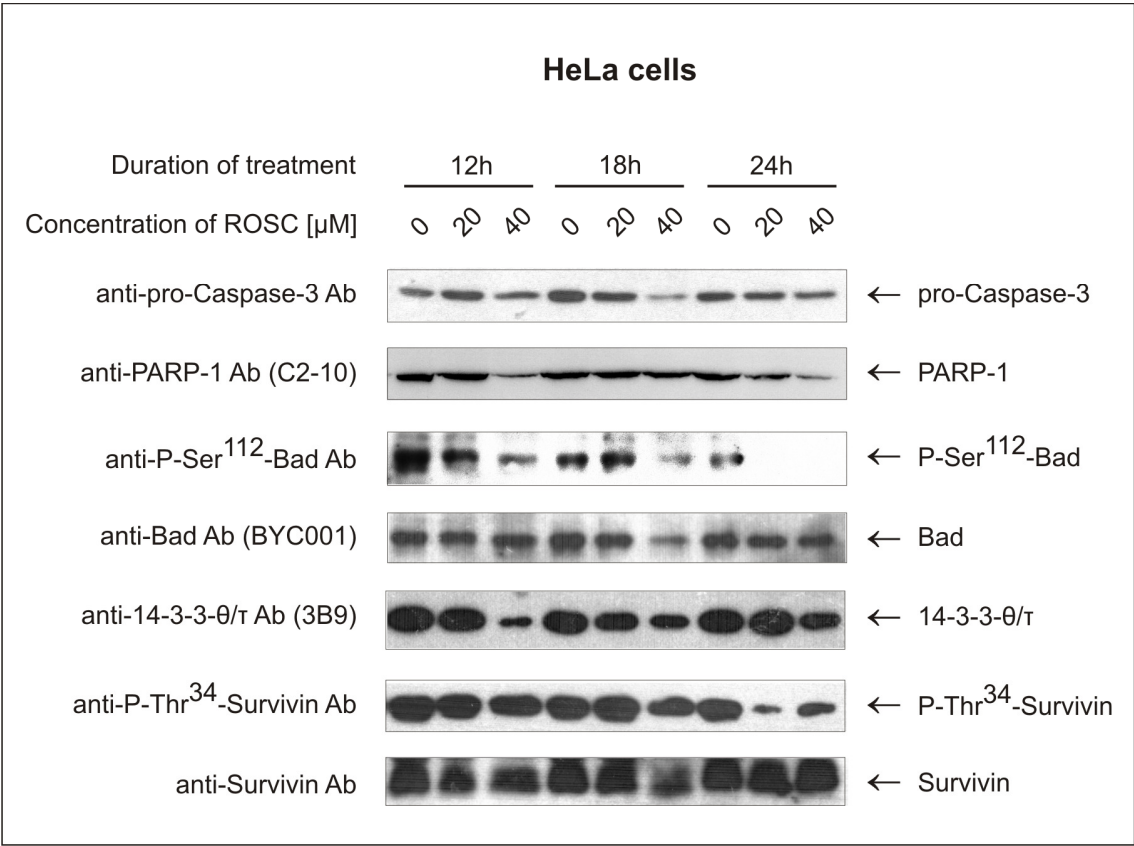


Fig. 66: ROSC affects several regulators of apoptosis. Control cells and ROSC-treated HeLa cells were lysed. Protein samples were analyzed by immunoblotting for the presence of major mediators of apoptosis like pro-Caspase-3, Bad or survivin.

The analyses revealed by which mechanisms ROSC promotes apoptosis in HeLa cells. Activation of selected pro-apoptotic proteins as well as repression of some pro-survival factors caused by modulation of their phosphorylation status in response to ROSC treatment preceded the onset of apoptosis.

3.4.6. Short-term ROSC treatment does not have any impact on apoptosis mediators in HTB-31 cells

Cell cycle analyses of HTB-31 cells demonstrated the complete absence, in the case of OLO II, or a delayed appearance of hypoploid cells in the case of ROSC. These findings were at least partially confirmed by the above depicted apoptotic assays. Additionally, HTB-31 cells were analyzed for the status of several apoptosis mediators after ROSC treatment.

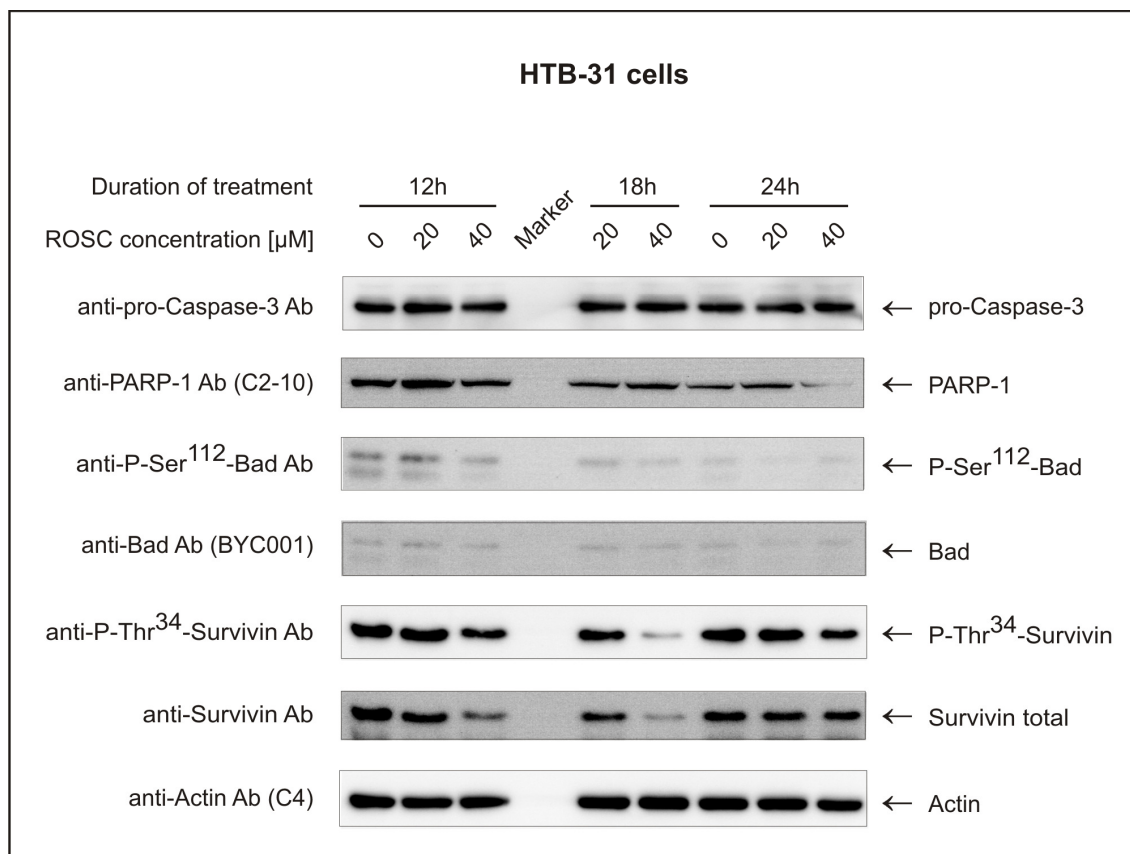


Fig. 67: ROSC has no effect on regulators of apoptosis in HTB-31 cells. HTB-31 cells were treated with ROSC at different concentrations and time points. Regulators of apoptosis were analyzed by immunoblotting.

Independent of the ROSC concentration used, HTB-31 cells did not show any temporal change in pro-caspase-3 levels, suggesting its inactive state (Fig. 67).^[Paper No. 1] Indeed, some modifications concerning PARP-1 cleavage were observed after 24h of treatment, a fact which was quite mysterious.

In addition, neither Bad nor survivin phosphorylation were affected by the ROSC treatment. However, Bad proteins seem to be expressed at low levels in HTB-31 cells.

In summary, ROSC does not display any apoptotic effects on HTB-31 cells, at least not during short treatment periods.

3.4.7. OLO II activates initiator and effector caspases in HTB-31 cells

Further investigations concerning the determination of caspase-9 activity lead us to two essential facts, which were already predicted earlier.

On the one hand results depicted in Fig. 68 clearly show that ROSC indeed does not affect apoptosis during short-term treatments in HTB-31 cells. However, it exerts temporally delayed apoptotic effects. During longer treatment periods ROSC induced accumulation of

caspase-9 was very effective and most notably when the medium was changed and cells were post-incubated in a drug-free medium. OLO II in contrast is even more potent than ROSC, thus triggering caspase-9 activation already after 24h of treatment very significantly. This effect is enhanced once again after 48h of treatment, being sixfold higher than in control cells.

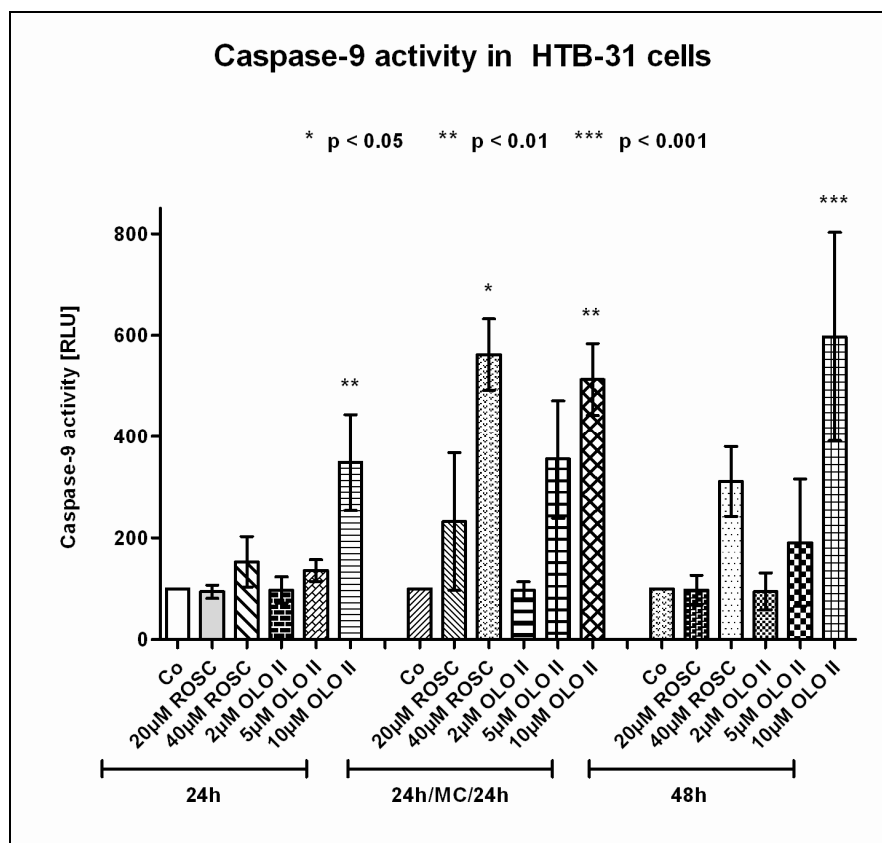


Fig. 68: OLO II leads to accumulation of caspase-9 in HTB-31 cells already after 24h of treatment. Control and drug-treated HTB-31 cells were investigated for caspase-9 activity using Caspase-Glo® 9 assay from Promega. The caspase-9 activity was stated as relative luminescence units (RLU) and normalized to the number of viable cells assessed by proliferation assays as earlier described.

Additional analyses of caspase-3/7 activity in human HTB-31 cells confirmed caspase-9 achieved results. Likewise, caspase-3/7 accumulation is induced only by OLO II already after 24h (Fig. 69). Longer treatment periods display an increase in caspase activation mediated by ROSC and OLO II, the latter being more efficient.

Interestingly, effector caspases were released to the culture medium already after 24h of OLO II treatment and increased over time to an eighteenfold amount than observed in controls. Even long-term effects of ROSC induced such a release, however at much lower extents.

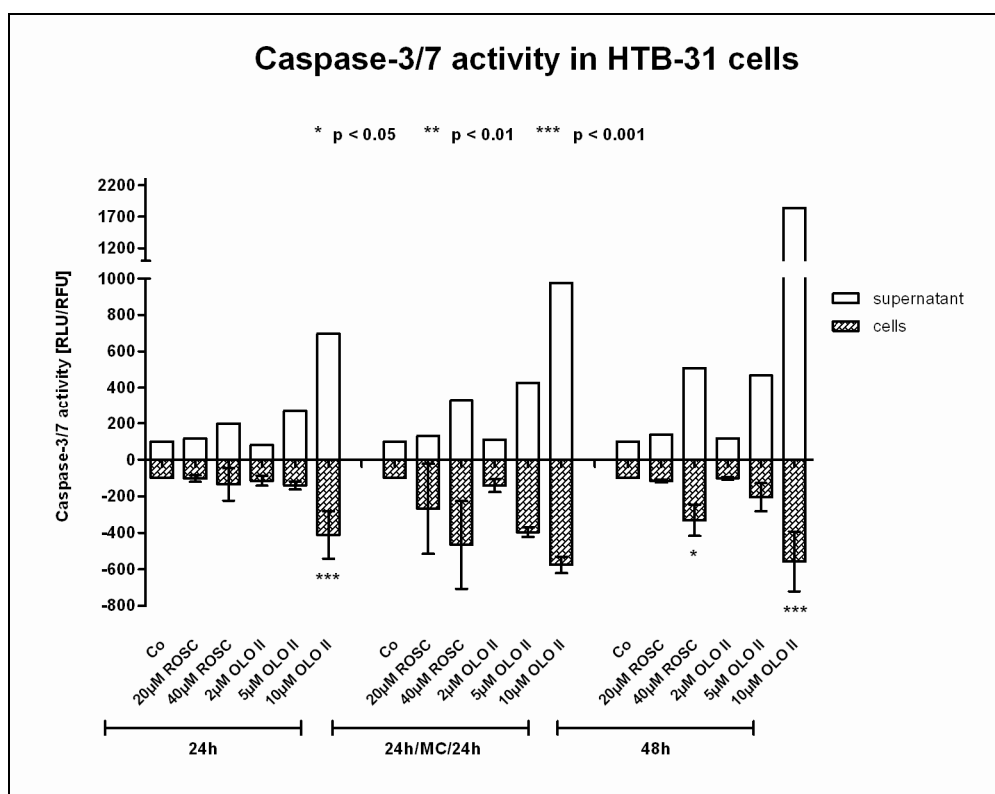


Fig. 69: Activation of effector caspases by OLO II in HTB-31 cells. HTB-31 cells were treated as indicated and tested for Caspase-3/7 activity either in cells or in their culture medium. Thereby used Caspase-Glo® 3/7 for cells and Apo-ONE® assay for supernatants were achieved from Promega. The caspase-3/7 activity was stated as relative luminescence units (RLU) or alternatively as relative fluorescence units (RFU) and normalized to the number of viable cells assessed by proliferation assays.

Caspase assays showed the higher resistance of HTB-31 cells towards certain anti-cancer drugs in contrast to HeLa cells. Nevertheless, HTB-31 cells were affected by OLO II or by ROSC after treatment for longer periods of time.

3.5. Synchronization of HeLa cells by mimosine and trichostatin A

All experiments performed so far were done on asynchronously growing HeLa and HTB-31 cells. This means that the cell population used for experiments comprises cells in distinct cell cycle phases, some of them being more proliferative than others and therefore faster in running through the cell cycle. In some cases, however, it might be of advantage to synchronize cells before the medication. Synchronization can be achieved by certain chemical compounds and refers to the process which retains cells in the same cell cycle phase at the same time.

Mimosine, a plant amino acid, and trichostatin A, an antibiotic, are known to have such synchronization effects in some cancer cell lines. Our ambition was to assess, whether HeLa cells could be synchronized by the latter and if this would provide an enhancement of ROSC treatment.

3.5.1. ROSC does not efficiently arrest cell cycle, when HeLa cells were previously synchronized by mimosine

As reported by Torsten Krude, HeLa cells can be synchronized with mimosine at a final concentration of 0.5 mM. ^[142]

Indeed, when treatment of HeLa cells was carried out with the specified MIMO concentration, a strong G₁-arrest (80 %) was observed (Fig. 70). This was accompanied by the reduction of the S phase population to one third, whereas the frequency of G₂ cell population remained unaffected. Once cells were released into a drug-free medium, they re-entered the cell cycle resulting in a transient accumulation of S phase cells. Surprisingly, after 12h of recovery the ratio of hypoploid cells increased. After 24h the initial distribution of cell cycle phases was re-established. Thus, MIMO-induced G₁ synchronization of HeLa cells is reversible. Shortly after removal of MIMO cell cycle progresses.

When MIMO-synchronized HeLa cells were released from the block into medium supplemented with ROSC, its effect differed from that achieved with asynchronously growing cells. ROSC at lower dose dramatically increased the population of S phase cells after 12h at the expense of G₁ cells. This effect was only transient since a treatment with ROSC for 24h resulted in a G₂ arrest. The higher ROSC concentration did not affect G₂ cell population, but re-established the initial distribution of cell cycle phases already after 12h. Thus, exposure of MIMO-synchronized cells to ROSC did not maintain the G₁ arrest. However, ROSC at the higher dosage increased the apoptosis rate to 60 %. Thus, our results showed that HeLa cells released from MIMO-mediated G₁ arrest respond to ROSC very similar to asynchronously growing cells except their stronger apoptotic capability. Therefore, synchronization of HeLa cells by MIMO prior to the onset of ROSC treatment provides very limited, if any, benefit.

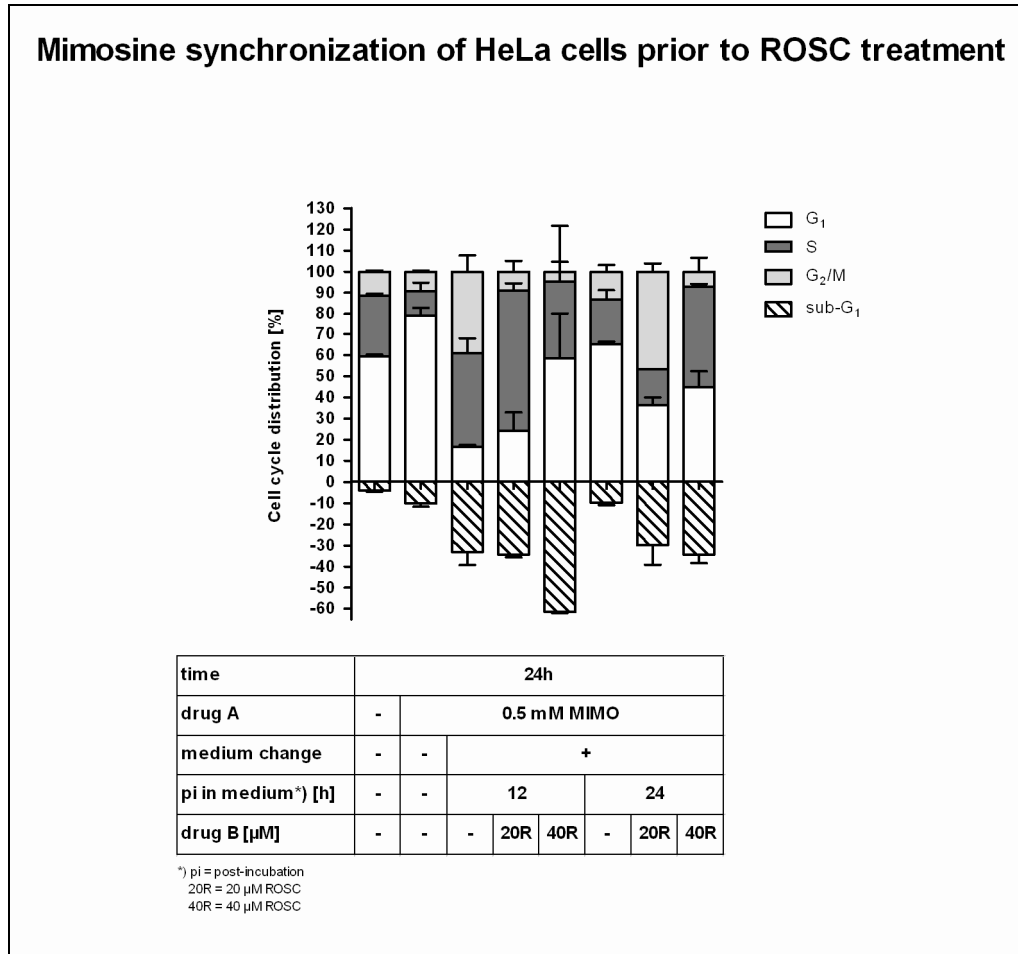


Fig. 70: ROSC did not maintain mimosine-induced G_1 block in HeLa cells. HeLa cells were first treated with 0.5 mM mimosine for 24h to synchronize cells. After a medium change cells were either incubated with or without ROSC at two different concentrations for the further 12h and 24h. Cells were subsequently harvested and DNA content was measured by flow cytometry.

3.5.2. ROSC enhances G_1 arrest in TSA-synchronized HeLa cells

Synchronization of HeLa cells with TSA, an inhibitor of histone deacetylases, leads to the hyperacetylation of chromatin, thus enhancing gene expression and affecting DNA replication at the same time. In most cases this correlates with a permanent activation of certain genes. [145, 146]

How the synchronization of HeLa cells by TSA prior to the ROSC treatment might affect the action of the latter was investigated in further experiments. HeLa cells were exposed for 24h to 400 ng/ml TSA. The medium was subsequently changed and cells were cultivated in the absence or presence of ROSC for the further 12h or 24h.

Inspection of cells under light microscopy revealed obvious changes of cellular morphology appearing after TSA treatment (Fig. 71). HeLa cells growing as an adherent culture have normally an oval shape, which changes during cellular division facilitating their de-

tachment from the substratum. A 24h treatment with TSA, however, completely altered their phenotype; they become spindle-shaped with multiple filopodia.

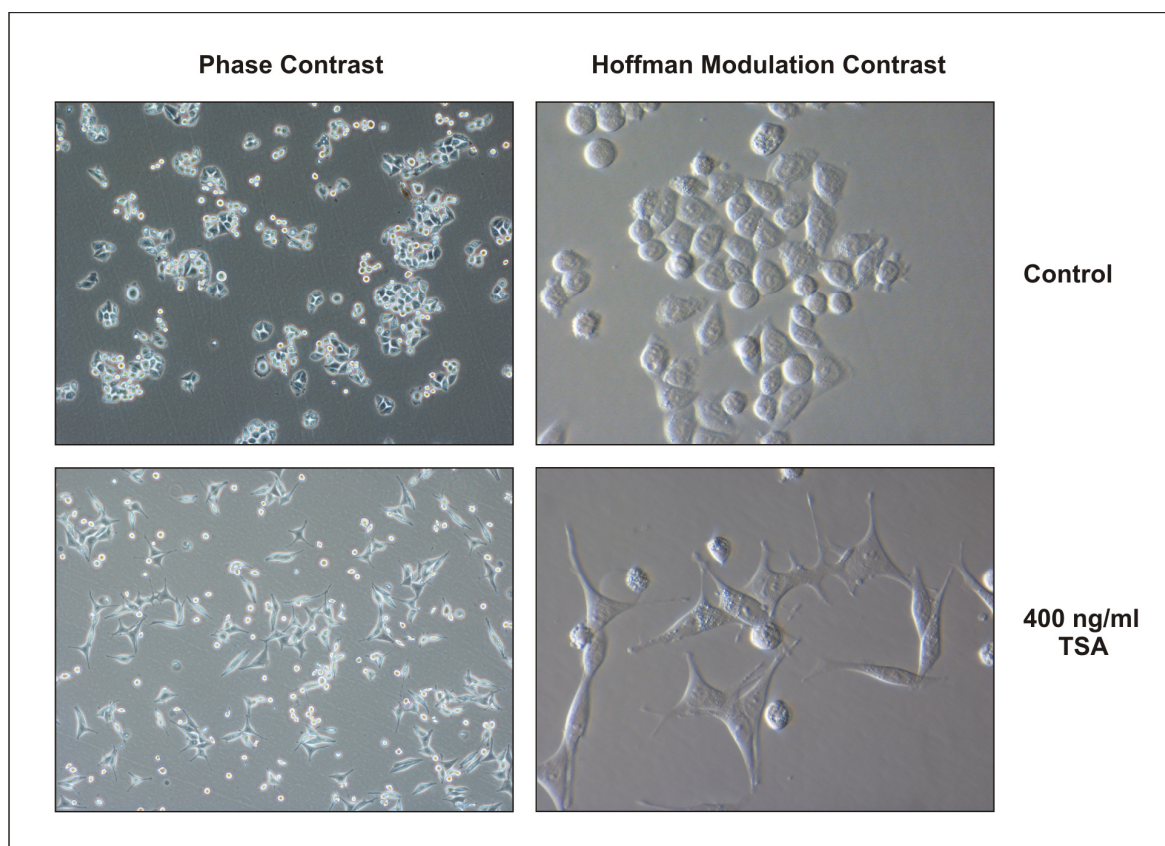


Fig. 71: TSA-induced morphological changes in HeLa cells after 24h of treatment. Images on the left were taken at the 10x magnification objective, those on the right were taken at 40x magnification objective and represent a three-dimensional view of cells by taking advantage of the Hoffman modulation contrast.

The analysis of the DNA concentration in single cells evidenced that TSA treatment arrested HeLa cells in G₁ and G₂ phase reducing simultaneously the ratio of the S phase cells (Fig. 72). Moreover, TSA induced apoptosis in approximately 20 % of cells. Thus, TSA led to modification of chromatin conformation. One might speculate that increase of acetylation enhanced expression of genes encoding pro-apoptotic proteins.

The TSA-induced cell cycle block was maintained even after cells were released into drug-free medium indicating that recovery of cells in the active cell cycle is a slow process. After post-incubation of cells in a drug-free medium, the ratio of hypoploid cells increased, reaching up to 50 % after 12h. In the next step, TSA-synchronized cells were released from the block in a drug-free medium or alternatively in medium containing ROSC. The short exposure (12h) of cells released from G₁ arrest to ROSC had a very weak effect on the distribution of cells in cell cycle. The ratio of apoptotic cells was reduced at 40 μ M by approximately 50 % as compared to the control. After exposure for 24h a dose-dependent

difference became evident. ROSC at a final concentration of 20 μM increased the frequency of S phase cells, whereas ROSC at 40 μM enhanced G_1 arrest that was associated with a decrease of S phase cells. Moreover, the rate of apoptotic cells increased by approximately 20 %.

In the next experimental series TSA-synchronized cells were released from the block in a drug-free medium for 1h and then ROSC was added. Interestingly, the maintenance of cells in a drug-free medium for 1h had very low impact on the action of ROSC. The trend was very similar to that observed after onset of treatment directly after release of TSA-arrested cells.

In summary, TSA synchronization of HeLa cells prior to the onset of ROSC treatment enhances G_1 arrest. However, increase of apoptosis rate seems to be ROSC-independent because in cells released from the block in a drug-free medium size of apoptotic population was comparable.

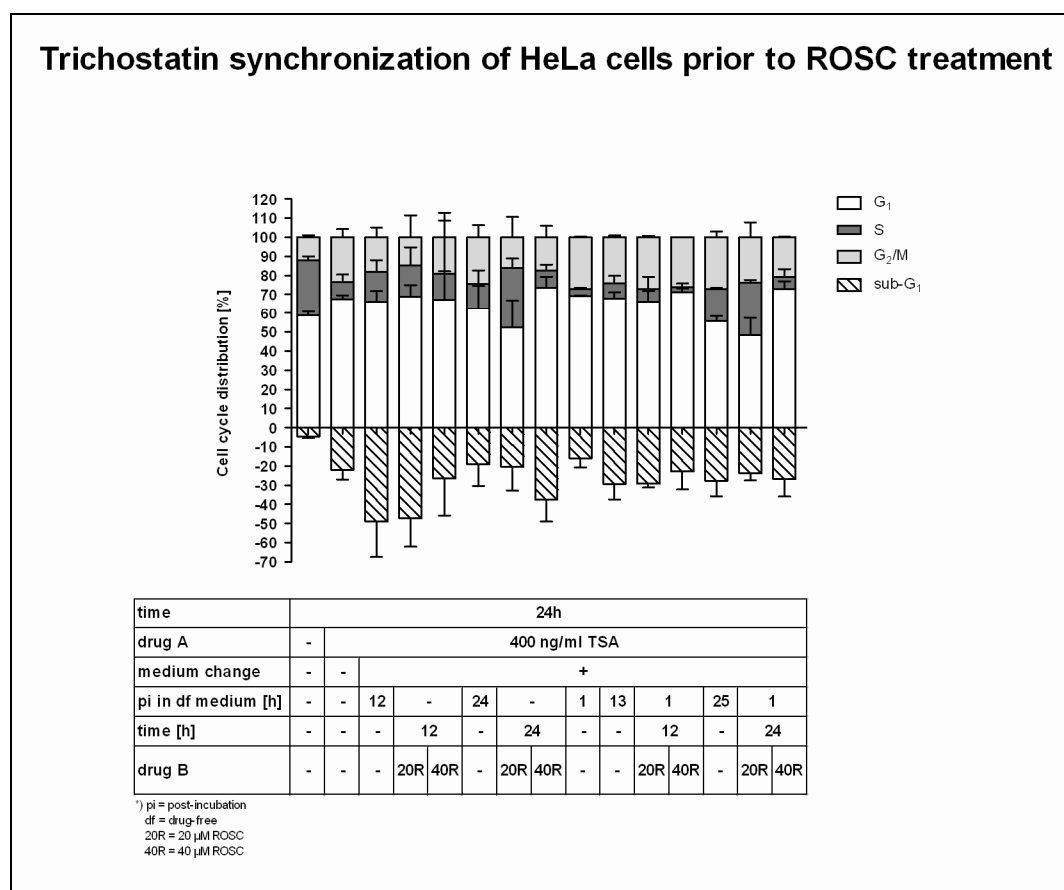


Fig. 72: Synchronization of HeLa cells by TSA prior to ROSC treatment slightly increases G_1 arrest. HeLa cells were synchronized for 24h with 400 ng/ml TSA. Thereafter the medium was changed and cells were incubated in the absence or presence of ROSC, as indicated. The treatment was done directly after release of cells from TSA-mediated block or after 1h of recovery. DNA content was determined by flow cytometry.

3.5.3. ROSC affects cell cycle and apoptosis regulators more efficiently, when HeLa cells were previously synchronized by TSA

Analyses of immunoblots further revealed that ROSC reduces the activating phosphorylation of CDK7 more efficiently when cells were synchronized with TSA prior to the treatment. The decrease of phosphorylated CDK7 occurred in a time- and concentration-dependent manner (Fig. 73). Already after 12h of ROSC treatment CDK7 phosphorylation was diminished and almost completely disappeared after 24h of 40 μ M ROSC. Remarkably, the activating phosphorylation of CDK2 activity was in contrast markedly reduced upon TSA treatment and a further incubation with ROSC had a very weak effect.

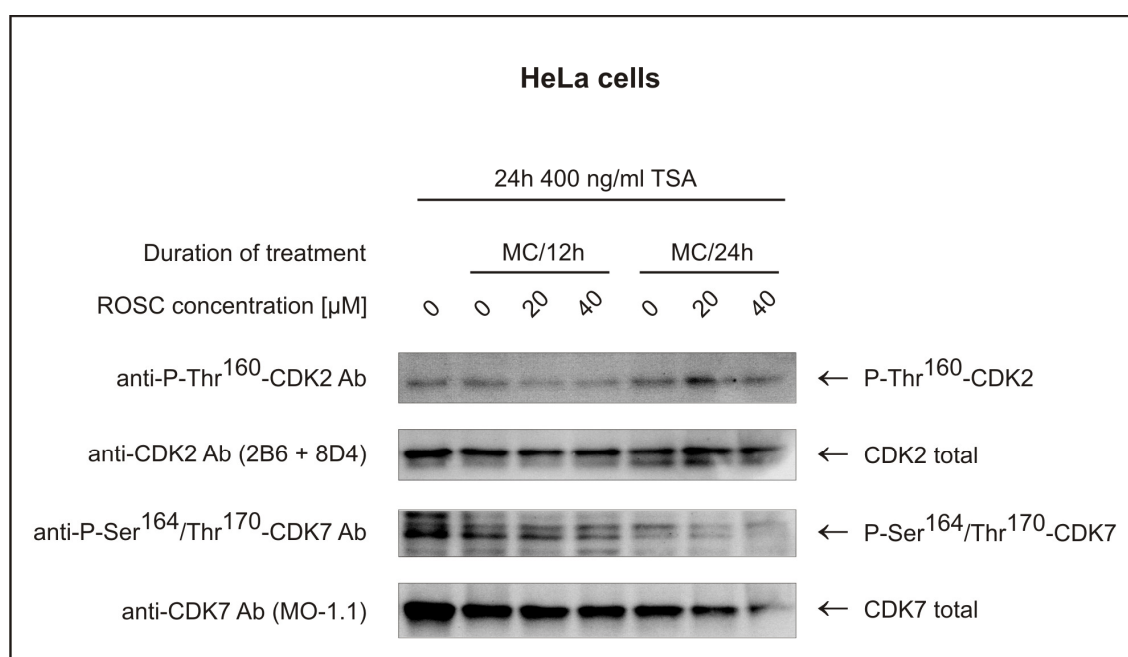


Fig. 73: Synchronization and ROSC treatment of HeLa cells affect CDK7 activity. HeLa cells were synchronized with 400 ng/ml TSA for 24h and after a medium change subsequently treated with ROSC. Immunoblots were probed for phosphorylation of CDK2 and CDK7 at activating sites.

The increased hyperacetylation of histones also affected the apoptosis regulators Bad and survivin following ROSC treatment. In the case of Bad protein, its phosphorylation at Ser¹¹² was reduced by the higher ROSC concentration already after 12h of treatment and almost abolished after further 12h (Fig. 74). At a lower concentration this effect became evident only after a longer incubation period. However, the phosphorylation of Bad was also reduced in controls after 24h.

Unfortunately, the cellular levels of total Bad protein were not proven due to technical problems.

Likewise, phosphorylation of survivin protein was affected by the same treatment. ROSC reduced its phosphorylation at Thr³⁴ in a time- and concentration-dependent manner. At

higher dose the decrease of site-specific phosphorylation of survivin became evident after 12h, which was additionally continued after 24h of treatment. As seen before, phosphorylation of survivin was also diminished in TSA-synchronized cells. Remarkably, the treatment decreased the cellular levels of total survivin protein, indicating that the phosphorylation determined stability of this protein.

These results clearly show that functional status of two examined regulators of apoptosis was more affected after ROSC treatment, when HeLa cells were synchronized prior to the onset of treatment.

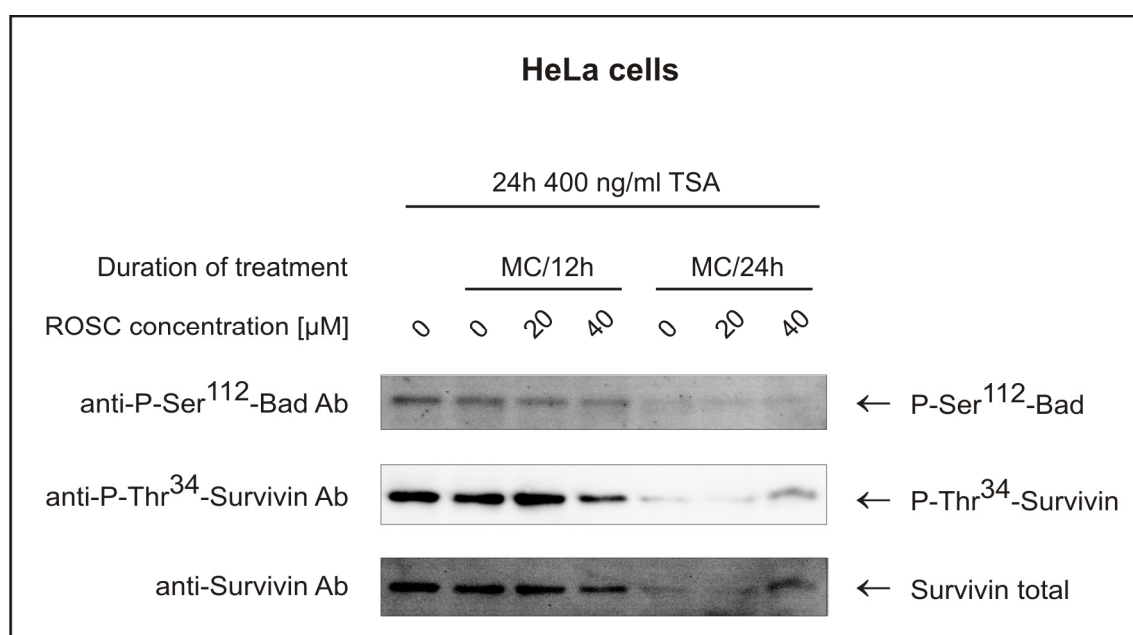


Fig. 74: Abrogation of phosphorylation of Bad and survivin after ROSC treatment of G₁-arrested HeLa cells. HeLa cells were synchronized and treated as usually. Obtained whole cell lysates were electrophoretically separated and blotted onto membranes, which were then analyzed by immunoblotting as indicated.

4. DISCUSSION

Deregulation of the proper cell cycle conferring enhanced dividing potential is a major hallmark of cancer cells. Moreover, cell death pathways governing the elimination of supernumerary or damaged cells are aberrantly regulated in transformed cells thereby promoting their survival. Thus, in most cancer types cellular factors controlling cell cycle progression and apoptosis are altered to overcome surveillance. This includes primarily constitutive activation or upregulation of cyclin-dependent kinases, overexpression and aberrant cytoplasmic localization of cyclins and inactivation of cellular CDK inhibitors. The amplified activity of CDKs and/or the concomitant inactivation or loss of cellular CDK inhibitors provided rationale to replenish this deficit by the use of pharmacological inhibitors to specifically target CDKs in a novel therapeutic approach. The application of such small-molecule inhibitory compounds might compensate the occurring deficiencies. Currently, very promising pharmacological CDK inhibitors are ROSC and especially OLO II. These purine analogues were the focus of interest of this diploma thesis.

The main purpose of research was to analyze to what extent pharmacological CDK inhibitors may affect proliferation and survival of human cervix carcinoma cells and to assess the relevance of the p53 status for the efficacy of the treatment. Therefore, two different human carcinoma cell lines, harbouring an inactivated p53 protein, were investigated. However, the way the inactivation is accomplished distinguishes both cell lines. Although HeLa cells harbour a wt *TP53* gene, its product is unfortunately not functional due to the expression of HPV-encoded E6 oncoprotein. E6 binds to p53 thereby sequestering it to cellular E6-AP ubiquitin E3 ligase complex and in this way targets it for ubiquitin-mediated degradation in the proteasome. Consequently, the G₁ restriction checkpoint is abrogated. In contrast to HeLa cells, a point mutation in the *TP53* gene in HTB-31 cells results in the accumulation of mutated p53 protein. The highly expressed mutant p53 protein in HTB-31 cells has no tumor suppressor activity, but it might promote cell survival since some point mutations of *TP53* gene are gain of function conferring it new functions.

Our results showed that HeLa as well as HTB-31 cells are definitely susceptible to the treatment with pharmacological CDK inhibitors. A marked reduction of the cell number after ROSC or OLO II treatment evidenced their strong anti-proliferative effects exerted on both cancer cell lines. However, observed effects are time and concentration dependent. ROSC and OLO II showed to be most effective in a discontinuous long-term treatment. This reflects their impact after *in vivo* administration and is of great therapeutical rele-

vance, since patients naturally degrade the administered drugs over time. Surprisingly, OLO II at a fourfold lower dose induced exactly the same effects as achieved by higher ROSC concentrations. Thus, OLO II is, despite its structure similarity to ROSC, more potent than the latter. This seems to be cell line-independent, since OLO II also eliminated HTB-31 cells more efficiently than ROSC. However, HTB-31 cells were generally more resistant to the action of pharmacological CDK inhibitors.

Further cell cycle analyses revealed the cell cycle modulatory as well as pro-apoptotic effects of both CDK inhibitors on HeLa and HTB-31 cells. DNA profiles illustrated the distribution of cell cycle phases in both examined cell lines after treatment and evidenced the concentration-dependent outcome. Low concentrations of both pharmacological CDK inhibitors arrested asynchronously growing HeLa cells already after 12h of treatment in the G₂/M phase. In contrast, higher doses accumulated a considerable hypoploid cell population (30 %), which most frequently represents cells undergoing apoptosis. Although the effects exerted by ROSC and OLO II were similar to those in HeLa cells, HTB-31 cells showed a delayed response to the treatment. Moreover, barely any apoptotic cells were detected. This confirms the relative resistant nature of HTB-31 cells to cell death.

Additional analyses of the pro-apoptotic effects of tested CDK inhibitors elucidated the mechanism by which apoptosis is triggered. The occurrence of a cleavage product of cytokeratin 18, a cytoskeleton protein and a typical downstream target of caspase-3, led to the assumption that caspases might be involved. This was further substantiated by detection of activated initiator and effector caspases. ROSC as well as OLO II induced elevated levels of caspase-9, which correlated with the increased activity of caspase-3/7 in HeLa cells. Likewise, HTB-31 cells were eliminated by caspase-mediated apoptosis, if treatment occurred long enough. Furthermore, extended treatment periods released activated caspases into the culture medium, indicating that the cell membrane becomes permeable. After staining of cells with the fluorescent JC-1 dye, it became also evident that ROSC and OLO II-treated cells fail to accumulate J-aggregates. This indicates the disruption of the electrochemical potential of the mitochondrial membrane with a subsequent collapse of the latter. Thus, CDK inhibitors induced apoptosis through the release of cytochrome c and promoted apoptosome formation. Moreover, ROSC-induced stabilization of the pro-apoptotic protein Bad and the concomitant abrogation of survivin phosphorylation, which is necessary for its stabilization, preceded the onset of apoptosis in HeLa cells.

Consequently, the results presented above clearly provide evidence for the bi-functionality of ROSC and OLO II, which might increase their therapeutical benefit.

Subsequent analyses of the functional status of major cell cycle regulators provided more details about the action of pharmacological CDK inhibitors. ROSC mainly affected CDK2 by preventing its activating phosphorylation at Thr¹⁶⁰ and CDK1 by increasing the phosphorylation of its inhibitory sites. Thus, inactivated CDK1 contributed to the accumulation of cells in G₂/M phase. Additionally, CDK7, a dual-specificity kinase involved in cell cycle regulation and transcription, was another target of ROSC. The CDK-activating kinase activity of CDK7 is active throughout the cell cycle. However, the kinase activity needed for transcription is positively regulated by the phosphorylation at Thr¹⁷⁰ and enhances the affinity of CDK7 for the RNA polymerase II. CDK7 is then able to phosphorylate Ser⁵ in the heptapeptide repeat of the CTD of RNA polymerase II and thus to induce initiation of transcription. ^[172] ROSC at higher concentrations was shown to diminish the site-specific phosphorylation of CDK7 at the aforementioned site thereby affecting the global transcription. This was demonstrated by the marked repression of the phosphorylation of Ser⁵ within the CTD of RNA polymerase II. A concomitant decrease of the phosphorylation of Ser² within the same heptapeptide repeat was additionally observed and correlated with the inability of RNA polymerase II to proceed in elongation and process of primary transcripts. We demonstrated that OLO II had similar effects on the global transcription in HeLa cells and HTB-31 cells, although being weaker in the latter.

Further analyses of the functional p53 status revealed that ROSC has different effects on this protein in HeLa and HTB-31 cells. In response to ROSC treatment a strong upregulation of p53 occurred in HeLa cells and was barely detectable in controls. This result indicates that the blockage of global transcription in HeLa cells upon treatment does not affect cellular p53 protein levels. Hence, reactivation of p53 is due to repression of the virally encoded E6 oncoprotein. Both CDK inhibitors were shown to efficiently decrease the expression of HPV-encoded E6 protein at higher concentrations. Subsequently, p53 protein is stabilized. One could speculate that increased and reactivated p53 protein contributes to the induction of apoptosis and cell cycle arrest.

However, the p53 reactivation appeared exclusively in HeLa cells. p53 levels in HTB-31 cells remained constant throughout the treatment period and demonstrated its prolonged stability. This constitutes an evidence for the inability of CDK inhibitors to overcome the mutation in this protein.

Interestingly, further experiments on HeLa cells emphasized that the outcome of treatment strongly depends on their cell cycle status prior to the onset of treatment. On the one hand, this was demonstrated by the fact that the recovery time of cells prior to the onset of treat-

ment influenced the efficacy of CDK inhibitors. On the other hand, experiments with synchronized HeLa cells released into a post-treatment evidenced enhancement of treatment in some cases. Our results showed that ROSC transiently enhanced the induced G₁ arrest in TSA-synchronized cells. This coincided with the outcome of nocodazole-arrested cells treated with ROSC. Thus, cells released from TSA as well as nocodazole-mediated block synchronized in early G₁ phase and provided a benefit for the treatment. In contrast, cells released from serum starvation or MIMO were synchronized in the late stage of G₁ phase and had no impact on ROSC treatment in HeLa cells.

In conclusion, ROSC and OLO II are both potent pharmacological CDK inhibitors, which are able to re-establish the proper cell cycle regulation in HeLa cervix carcinoma cell lines. This is attributed to their pleiotropic effects described above. However, the success of the treatment with ROSC or OLO II depends on multiple factors, including the duration of therapy, the administered drug concentration and the cell cycle status prior to the administration. Both pharmacological CDK inhibitors have also pro-apoptotic effects with a concomitant low cytotoxicity towards cells. In addition, it was proven that, unlike other drugs (DOX or CP), ROSC and OLO II do not damage cellular DNA.^[173] This fact makes both CDK inhibitors very interesting for targeted therapies. Due to their high selectivity for transformed cells ROSC and OLO II may be better tolerated than conventional chemotherapeutic drugs. Further investigations of both CDK inhibitors might lead to successful anti-cancer drugs with low side-effects.

APPENDIX

I. List of chemicals or other materials

Centrifuge 3K30	Sigma
Centrifuge 5417C	Eppendorf
Chemi-Smart™ 5100	Peqlab Biot. GmbH
Combitips plus (2.5 ml, 5 ml)	Eppendorf
Cuvettes 1.5 ml	Bio-Rad
Digitonine	Sigma
Doxorubicin	Haiman Pharmaceutical Factory
Eclipse TE300 inverse microscope	Nikon
Eppendorf tubes 0.6 ml, 1.5 ml, 2 ml	Eppendorf
Falcons 15 ml, 50 ml (sterile)	Greiner Bio-One
HCl	Carl Roth GmbH
Marker, pre-stained benchmark protein ladder	Gibco BRL
Microplate Reader, Synergy HT	BioTek
Mimosine	Sigma
Multilabel-Multitask Plate Counter (Wallac 1420 Victor)	Beckman
Multipette plus	Eppendorf
NaF	Merck
NaOH	Carl Roth GmbH
NaVO ₃	Sigma
Olomoucine II	was synthesized and provided within a FWF project by Prof. Dr. M. Strnad and Dr. V. Krysstof from Palacký University, Olomouc, Czech Republic
Potter homogenizer	
Power supply, PowerPac 200	Biorad
Roscovitrine	was synthesized and provided within a FWF project by Prof. Dr. M. Strnad and Dr. V. Krysstof from Palacký University, Olomouc, Czech Republic

Round-bottom tubes 5 ml for flow cytometry

Sonicator, Sonoplus GM70

Spectrophotometer, U 2000

Streptavidin

X-ray film processor, Typ 1140

Becton Dickinson

Bandelin

Hitachi

Protec Gerätebau

II. List of used antibodies

A. Primary antibodies

14-3-3- θ/τ (Clone 3B9)	Sigma
Actin (Clone C4)	MP Biomedicals LLC
P-Ser ¹¹² -Bad	Millipore
Bad (Clone BYC001)	Millipore
pro-Caspase-3	DAKO Corp.
P-Ser ²¹⁶ -cdc25C	Cell Signaling
cdc25C	Cell Signaling
P-Thr ¹⁶¹ -CDK1	Cell Signaling
P-Thr ¹⁴ /Tyr ¹⁵ -CDK1	Sigma
CDK1	Cell Signaling
P-Thr ¹⁶⁰ -CDK2	Cell Signaling
CDK2 (Clone 2B6 + 8D4)	Neomarkers
P-Ser ¹⁶⁴ /Thr ¹⁷⁰ -CDK7	Biolegend
CDK7 (Clone MO-1.1)	Sigma
CytoDEATH M30-fluoresceine-coupled antibody	Roche Diag. GmbH
E6 (Clone BF7)	Abcam
E7	Abcam
P-Ser ⁹⁰⁹ -LATS-1	Cell Signaling
LATS-1	Cell Signaling
P-Thr ¹⁹⁹ -NPM	Cell Signaling
NPM (Clone Ab-1)	Neomarkers
P-Ser ⁵³⁶ -NF κ B	Cell Signaling
NF κ B (p65)	Cell Signaling
PARP-1 (Clone C2-10)	from Dr. Guy Poirier, Laval University of Quebec
PCNA (Clone PC-10)	Calbiochem
Ran (Clone 20)	BD Transd. Lab.
P-Ser ⁷⁸⁰ -Rb	Cell Signaling
P-Ser ^{807/811} -Rb	Cell Signaling
Rb (Clone IF-8)	Santa Cruz Biotech.
P-Ser ² -RNA Polymerase II (Clone H5)	Abcam

P-Ser⁵-RNA Polymerase II (Clone H14)

RNA Polymerase II

p53 (Clone DO-1)

P-Thr³⁴-Survivin

Survivin

Abcam

Alexis

was a kind gift from
Dr. Vojtesec

Biolegend

Santa Cruz Biotech.

B. Secondary antibodies

anti-mouse IgG, peroxidase conjugated

anti-rabbit IgG, peroxidase conjugated

anti-mouse IgM, biotin conjugated

Pierce

Sigma

Amersham Biosc.

III. List of Figures

Figure 1:	Schematic view of cell cycle stages	16
Figure 2:	Regulation mechanisms of CDKs	19
Figure 3:	Cyclin expression during cell cycle	20
Figure 4:	Cell cycle checkpoints restricted to defined spots	21
Figure 5:	a. General MAPK signal transduction pathway step by step, b. Raf-MEK/1/2-ERK1/2 pathway in detail	24
Figure 6:	Cyclin-CDK complexes responsible for cell cycle progression	25
Figure 7:	Caspase zymogens forming heterodimers when activated	26
Figure 8:	Schematic view of the extrinsic and the intrinsic apoptosis pathway	27
Figure 9:	Properties of cancer cells	31
Figure 10:	General genome structure of human papilloma viruses	37
Figure 11:	HPV infection pathway of keratinocytes	37
Figure 12:	E6 and E7 oncogene interaction with important cellular tumor suppressor proteins	40
Figure 13:	Pap smear of cervical cells	42
Figure 14:	HeLa cells in cell culture flasks	47
Figure 15:	HTB-31 cells in cell culture flasks	48
Figure 16:	Molecular structure of doxorubicin hydrochloride	48
Figure 17:	Molecular structure of OLO II	49
Figure 18:	a. Crystal structure of CDK2 in complex with (R)-ROSC, illustrating the po- sition of (R)-ROSC in the ATP-binding pocket, b. Atoms involved in H-bond (R)-ROSC-CDK2 binding	50
Figure 19:	Molecular structure of mimosine	50
Figure 20:	Molecular structure of trichostatin A	51
Figure 21:	Luciferase reaction	54
Figure 22:	Caspase-Glo [®] 9 Assay	57
Figure 23:	Apo-ONE assay principle	58
Figure 24:	Dye exclusion test principle using 7-AAD as fluorescent dye	60
Figure 25:	Cytokeratin 18 cleavage by caspases during apoptosis	61
Figure 26:	a. Western Blot assembly, b. Position of gel and membrane regarding the poles in a blotting cell	71

Figure 27:	a. Chemiluminescence reaction of HRP coupled secondary antibodies,	
	b. Immunodetection using Biotin coupled secondary antibodies and streptavidin	72
Figure 28:	Dose-response curve for ROSC	79
Figure 29:	Dose-response curve for ROSC upon varying the exposure time	80
Figure 30:	Statistical significances of the reduction of the number of living cells after ROSC treatment	81
Figure 31:	Dose-response curves for OLO II	82
Figure 32:	Dose-response curve for OLO II after varying the exposure time	83
Figure 33:	Statistical significances of the diminution of the number of viable cells upon treatment by OLO II	84
Figure 34:	Comparison of treatment of HeLa cells with ROSC, OLO II and DOX	84
Figure 35:	Dose-response curve for the action of ROSC in HTB-31 cells	86
Figure 36:	Dose-response curve for OLO II in HTB-31 cells	86
Figure 37:	Significances of OLO II treatment of HTB-31 cells	87
Figure 38:	Comparison of the efficacy of the ROSC and OLO II action with that of DOX in human HTB-31 cells	88
Figure 39:	DNA profiles of control and ROSC-treated HeLa cells	89
Figure 40:	Distribution of cell cycle phases in HeLa cells after ROSC treatment	90
Figure 41:	Distribution of cell cycle phases in HeLa cells after OLO II treatment	91
Figure 42:	DNA Profiles of HTB-31 cells treated with ROSC for 36h and 48h	92
Figure 43:	Distribution of HTB-31 cells in distinct cell cycle phases after ROSC treatment	93
Figure 44:	DNA profiles of control HTB-31 cells and cells treated with OLO II for the indicated time points	93
Figure 45:	Distribution of HTB-31 cells in distinct cell cycle phases after OLO II treatment	94
Figure 46:	Abolishing of CDK2 and CDK1 activating phosphorylation after ROSC treatment of HeLa cells	96
Figure 47:	Decrease of NPM phosphorylation is an evidence for CDK2 inactivation by ROSC	96
Figure 48:	Cellular levels and phosphorylation status of cdc25C phosphatase and LATS-1 tumor suppressor protein in control and ROSC-treated HeLa cells	97
Figure 49:	Reduction of the site-specific pRb phosphorylation	98

Figure 50:	Abolishing of site-specific phosphorylation of CDK7 in response to ROSC treatment	99
Figure 51:	ROSC and OLO II abolish the phosphorylation of RNA polymerase II at specific serine residues critical for regulation of transcriptional progression .	100
Figure 52:	Increasing levels of p53 protein after ROSC treatment	101
Figure 53:	Repression of E7 oncoprotein by ROSC	102
Figure 54:	Repression of E6 oncoprotein expression by ROSC and OLO II	102
Figure 55:	Effect of ROSC and OLO II on the functional status of CDK7 and CDK2 in human HTB-31 cells	103
Figure 56:	Repression of cellular transcription in HTB-31 cells by OLO II	104
Figure 57:	ROSC and OLO II treatment does not affect levels of p53 protein in HTB-31 cells	105
Figure 58:	ROSC enhanced site-specific phosphorylation of NF- κ B in human HTB-31 cells in a time- and concentration-dependent manner	106
Figure 59:	Detection of caspase-3-cleaved cytokeratin 18 in human cervix carcinoma cells exposed to drugs	108
Figure 60:	Accumulation of monomeric JC-1 dye upon collapse of mitochondrial membrane after OLO II and DOX treatment	110
Figure 61:	Loss of J-aggregate formation upon treatment of HeLa cells	111
Figure 62:	Loss of J-aggregate formation upon treatment of HTB-31 cells	112
Figure 63:	Uptake of 7-AAD is an evidence for the loss of the membrane integrity ..	113
Figure 64:	Activation of initiator caspase-9 in control and drug-treated HeLa cells ..	114
Figure 65:	Enhancement of caspase-3/7 activity after treatment of HeLa cells with CDK inhibitors	115
Figure 66:	ROSC affects several regulators of apoptosis	117
Figure 67:	ROSC has no effect on regulators of apoptosis in HTB-31 cells	118
Figure 68:	OLO II leads to accumulation of caspase-9 in HTB-31 cells already after 24h of treatment	119
Figure 69:	Activation of effector caspases by OLO II in HTB-31 cells	120
Figure 70:	ROSC did not maintain mimosine-induced G ₁ block in HeLa cells	122
Figure 71:	TSA-induced morphological changes in HeLa cells after 24h of treatment	123
Figure 72:	Synchronization of HeLa cells by TSA prior to ROSC treatment slightly increases G ₁ arrest	124
Figure 73:	Synchronization and ROSC treatment of HeLa cells affect CDK7 activity	125

Figure 74: Abrogation of phosphorylation of Bad and survivin after ROSC treatment of G ₁ -arrested HeLa cells	126
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IV. List of Tables

Table 1:	Short résumé of HeLa S ₃ cell characteristics	46
Table 2:	Short résumé of HTB-31 cell characteristics	47
Table 3:	List of drug stock solutions and their concentrations	53
Table 4:	List of added volumina of drug stock solutions	54
Table 5:	Protein determination – pipetting scheme	67
Table 6:	SDS-PAGE – ingredients for stacking and separation gel	69
Table 7:	Reduction of the number of viable cells after treatment with examined drugs	85

V. Abbreviations

7-AAD	7-Aminoactinomycin D
APC	Anaphase-promoting complex
APS	Ammonium persulfate
ATM	Ataxia telangiectasia mutated
ATR	AMT- and Rad3-related
BSA	Bovine serum albumin
CAK	CDK-activating kinase
Caspase	Cystein aspartic acid protease
CCD camera	Charge-coupled device camera
CDK	Cyclin-dependent kinase
c_E	Endconcentration
Chk1/2	Checkpoint kinase 1/2
Cip	CDK inhibitor protein
CP	Cisplatin
DAPI	4', 6-Diamidino-2-phenylindole
ddH ₂ O	bi-distilled water
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin (= adriamycin)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FITC	Fluorescein
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IC ₅₀	Inhibitory concentration of a drug, which kills 50 % of alls cells
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
Kip	Kinase inhibitor protein
OLOII	Olomoucine II /2- {[2-((1- <i>R</i>)-1-hydroxymethyl-propylamino)-9-isopropyl-9 <i>H</i> purin-6-ylamino]-methyl}-phenol/

MC	Medium change
MeOH	Methanol
MIMO	L-Mimosine, (<i>S</i>)- α -Amino- β -[1-(3-hydroxy-4-oxopyridine)]propionic acid
Na-Doc	Natrium deoxycholic acid
NaF	Natrium fluoride
NaN ₃	Natrium acid
NaOH	Natrium hydroxid
NaVO ₃	Natrium vanadate
NLS	Nuclear localization signal
NP-40	Nonidet P-40
PBS	Phosphate buffered saline
Pefabloc® SC	4-(2-Aminoethyl)-benzolsulfonylfluorid-hydrochlorid
PMSF	Phenylmethysulfonylfluoride
pRb	Retinoblastoma protein
PVDF	Polyvinylidendifluoride
RIPA	Radioimmuno precipitation assay buffer
RNA	Ribonucleic acid
ROSC	Roscovitine [(2- <i>R</i>)-2-(6-benzylamino-9-isopropyl-9 <i>H</i> purin-2-ylamino)-butan-1-ol)]
RSB	Reticulocytes suspension buffer
Ser	Serine
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TEMED	Tetramethylethyldiamine
Thr	Threonine
Tris	Tris base, tris(hydroxymethyl)aminomethane
TSA	Trichostatin A R-(<i>E,E</i>)]-7-[4-(Dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide
Tyr	Tyrosine

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Title of qualification awarded	Magistra rerum naturalium (Mag. rer. nat.)
Principal subjects	Study of Genetics & Microbiology main subject – immunology secondary subjects – oncology and microbial ecology optional laboratory courses – organic chemistry, computer-based sequence and structure analyses, molecular parasitology, fluorescence in-situ hybridization, molecular biology of cyanobacteria, biochemical studies of the mechanism of carcinogenesis
Name and type of organisation providing education and training	Faculty of Life Sciences, University of Vienna
Dates	Sept. 1994 – June 2002
Title of qualification awarded	School-certificate examination, qualification for university attendance
Principal subjects	German, English, Latin, Mathematics and main focus on Biology
Name and type of organisation providing education and training	Bundesrealgymnasium Gottschalkgasse 21, 1110
Dates	Sept. 1990 – June 1994
Name and type of organisation providing education and training	Elementary school, Vienna

Work experience

Dates	May 2008 – June 2009
Occupation or position held	Diploma student
Main activities and responsibilities	Cell culture Testing of different anti-cancer drugs Different cell- and molecular biological techniques (various viability and apoptosis assays, PI staining for FACS, immunfluorescence stainings, cell fractionation, SDS-PAGE, Western Blots, autoradiografic immunodetection or with ChemiSmart imaging system) Statistical evaluation and interpretation of results
Name and address of employer	A.o. Univ. Prof. Dr. Józefa Gądek-Węsierska Institute of Cancer Research Department of Internal Medicine I Medical University of Vienna Borschkegasse 8a, 1090 Vienna, Austria
Type of business or sector	Cell Cycle Regulation Group
Dates	Oct. 2000 – June 2008
Occupation or position held	Private tutor
Main activities and responsibilities	Preparing pupils between 5 th and 12 th grade (gymnasium or realgymnasium) for final exams in subsequent subjects: German, English, Mathematics, Latin and Biology
Name and address of employer	Private lessons
Dates	Nov. 2003 – Dez. 2003
Occupation or position held	Sales assistant
Main activities and responsibilities	Sales, consultation and stock responsibilities
Name and address of employer	Benetton Megastore Vienna S&W Textilhandel GmbH Mariahilferstraße 116 1070 Vienna, Austria
Type of business or sector	Fashion store

Personal skills and competences

Mother tongue **Romanian**

Other languages **German, English, Italian**

Self-assessment

German

English

Italian

Understanding	Speaking	Writing
Proficient user		
Independent user		
Just basic understanding		

Social skills and competences	Excellent team spirit, experiences in interacting with different people, reliable
Organisational skills and competences	hard-working, accurate, punctual, sense of organisation through the long-time experience with private lessons
Computer skills and competences	Microsoft Office (Word, Excel, PowerPoint), Adobe Photoshop (basic skills), CorelDraw, GraphPad Prism, World Wide Web, good handling of molecularbiological database, some experience in Bioinformatics (basic sequence and structure analyses)

Wien, am 5. November 2009

Borza Andreea

Outcome of Treatment of Human HeLa Cervical Cancer Cells With Roscovitine Strongly Depends on the Dosage and Cell Cycle Status Prior to the Treatment

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ABSTRACT

Exposure of asynchronously growing human HeLa cervical carcinoma cells to roscovitine (ROSC), a selective cyclin-dependent kinases (CDKs) inhibitor, arrests their progression at the transition between G₂/M and/or induces apoptosis. The outcome depends on the ROSC concentration. At higher dose ROSC represses HPV-encoded E7 oncoprotein and initiates caspase-dependent apoptosis. Inhibition of the site-specific phosphorylation of survivin and Bad, occurring at high-dose ROSC treatment, precedes the onset of apoptosis and seems to be a prerequisite for cell death. Considering the fact that in HeLa cells the G₁/S restriction checkpoint is abolished by E7, we addressed the question whether the inhibition of CDKs by pharmacological inhibitors in synchronized cells would be able to block the cell-cycle in G₁ phase. For this purpose, we attempted to synchronize cells by serum withdrawal or by blocking of the mitotic apparatus using nocodazole. Unlike human MCF-7 cells, HeLa cells do not undergo G₁ block after serum starvation, but respond with a slight increase of the ratio of G₁ population. Exposure of G₁-enriched HeLa cells to ROSC after re-feeding does not block their cell-cycle progression at G₁-phase, but increases the ratio of S- and G₂-phase, thereby mimicking the effect on asynchronously growing cells. A quite different impact is observed after treatment of HeLa cells released from mitotic block. ROSC prevents their cell cycle progression and cells transiently accumulate in G₁-phase. These results show that inhibition of CDKs by ROSC in cells lacking the G₁/S restriction checkpoint has different outcomes depending on the cell-cycle status prior to the onset of treatment. *J. Cell. Biochem.* 106: 937–955, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: APOPTOSIS; CELL CYCLE ARREST; CYCLIN-DEPENDENT KINASES; INHIBITORS OF CYCLIN-DEPENDENT KINASES; ROSCOVITINE

Altered expression of cell cycle regulatory genes contributes to uncontrolled proliferation of malignant cells. Cyclin-dependent kinases (CDKs), the key enzymes governing progression through the cell cycle, are subject of frequent deregulation through genetic or epigenetic mechanisms providing the cells selective growth advantage [Malumbres and Barbacid, 2005, 2007]. These enzymes are composed of a catalytic kinase subunit and a regulatory cyclin subunit that is essential for activation of CDKs. Cyclins themselves are expressed only during particular cell cycle periods, allowing activation of individual CDKs at the right time. Besides, CDKs are regulated by stimulating phosphorylation of the activation

segment catalyzed by CDK-activating kinase (CAK), by inhibitory phosphorylation within the ATP-binding site induced by Wee/Myt kinases, and finally by their interactions with small protein inhibitors [Cip/Kip or INK4] [Malumbres and Barbacid, 2005; Besson et al., 2008]. All these proteins cooperate in a balanced network in normal cells, whereas abnormal CDK regulation is a hallmark of cancer cells. CDKs are mutated in cancers only seldom, with the rare exception of a point mutation in CDK4, resulting in the loss of INK4 binding, or more often by gene amplifications and overexpression of both CDK4 and CDK6 [Malumbres and Barbacid, 2005]. However, a significant number of cancers bear changes in

Abbreviations used: CAK, cyclin-dependent kinase-activating kinase; CDK, cyclin-dependent kinase; Cip/Kip, CDK-interacting protein/kinase-inhibiting protein; HPV, human papilloma virus; INK4, inhibitor of CDK4; NOC, nocodazole; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; pRb, retinoblastoma protein; ROSC, roscovitine; Waf1, wild-type p53-activated fragment 1.

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genes encoding CDK regulatory partners, including cyclins E and D, Cip/Kip and INK4 inhibitors, and their substrates (mainly retinoblastoma protein, pRb) [Malumbres and Barbacid, 2007; Besson et al., 2008]. This permits escape from senescence during malignant transformation and allows cancer cells to proliferate with increased CDK activity and accumulate further mutations.

On the basis of deregulated activity of CDKs detected in cancers, inhibitors targeting specifically these kinases have been developed as anticancer therapeutics. A growing number of small molecule inhibitors are now in preclinical testing or even in clinical trials [Fischer and Lane, 2000; Dai and Grant, 2003; Malumbres et al., 2008]. The most success so far has been obtained with ATP-mimicking competitive inhibitors of CDK. They are usually classified according to their specificities as pan-specific (do not discriminate among different CDKs) and oligo-specific (preferentially inhibit only some CDKs). PD-0332991, which has been developed to preferentially target CDK4/6, is a typical example of the latter group [Fry et al., 2004]. However, the majority of developed drugs, including flavopiridol, roscovitine (ROSC), or SNS-032, hit simultaneously multiple CDKs. These oligo-specific compounds potentially arrest the cell cycle in transformed cells, due to inhibition of cell cycle regulating CDK1, CDK2, and/or CDK4, and induce apoptosis as well, apparently as a result of transcriptional perturbations caused by CDK7 and CDK9 inactivation [Shapiro, 2006].

Interestingly, several pharmacological CDK inhibitors also potentially block transcription and replication of some viruses, such as HIV, HCMV, VZV, HSVs, and EBV, that rely on the activity of endogenous CDKs in host animal cells [Schang et al., 2005]. Some viruses can inactivate pRb through direct binding of viral proteins and the consequent abrogating of the G₁ restriction point, the others are able to constitutively activate cellular CDKs by virally encoded cyclin proteins [Helt and Galloway, 2003], or even encode kinases able to substitute CDKs in their ability to phosphorylate pRb [Hume et al., 2008]. It is assumed that the antiviral activity of CDK inhibitors is based on interference with viral transcription. Importantly, as some human cancers are induced by oncogenic viruses, like Kaposi's sarcoma or HPV-induced cervical carcinoma, therapy based on CDK inhibitors would not only stop the proliferation of transformed cells, but also advantageously limit the life-cycle of the transforming viruses, both dependent on cellular CDKs.

Human papilloma viruses (HPV) are small double-stranded DNA viruses infecting squamous epithelia, inducing proliferative lesions within infected tissues that are prerequisite for the development of cancer. The high risk HPV-encoded proteins E6 and E7, substantially contributing to transforming capability, play a major role in the deregulation of the cell cycle control. Protein E6 binds p53 and directs it to ubiquitin-mediated degradation, further promoting genetic instability in infected cells [Scheffner et al., 1990]. E7 not only induces aberrant S-phase entry through the inactivation of pRb tumor suppressor and related pocket-proteins, but also increases expression of E2F-responsive cell cycle regulators such as the cdc25a tyrosine phosphatase that further contribute to G₁/S deregulation [Nguyen et al., 2002]. Moreover, E7 prevents p21^{Waf1} both from inhibiting activity of CDK2/cyclin E and DNA replication and thus disrupts normal cell cycle control by increased CDK2 activity and enhanced expression of E2F-responsive genes [Funk

et al., 1997]. As shown in mouse models, inactivation of pRb by E7 is not sufficient to overcome G₁ restriction point; also other E2F regulators are important targets of E7 that play critical roles in cervical carcinogenesis [Balsitis et al., 2006]. For example, recent experiments demonstrated that E7 can associate with and inactivate the transcriptional repression activity of E2F-6, thereby extending S-phase competence of HPV infected cells [McLaughlin-Drubin et al., 2008]. Down-regulation of both viral oncoproteins increases the efficacy of the therapy [Wesierska-Gadek et al., 2002].

In the light of the facts mentioned above, the question appeared whether the inhibition of cellular CDKs by pharmacological inhibitors would be able to reduce the proliferation rate of cancer cells infected with high risk HPV and to restore the proper cell cycle control as well as to optionally trigger them to undergo apoptosis. For this purpose we decided to apply ROSC, a relatively selective CDK inhibitor targeting not only kinases regulating cell cycle progression but also CDK7, the dual acting kinase that is also implicated in the regulation of transcription [Fisher, 2005]. Considering the fact that cellular RNA polymerase II is required for transcription of HPV-encoded proteins and that CDK7 complexed with cyclin H is a constituent of the basal transcription factor TFIIF, which phosphorylates the serine residues within the heptapeptide repeat of the carboxy terminal domain (CTD) of RNA polymerase II, one might expect that ROSC would not only affect the functionality of the intrinsic cellular cell cycle regulators but would also prevent transcription of genes encoded by the infecting pathogen. We chose human HeLa cervical carcinoma cells, the most frequently investigated cell line derived from HPV-positive cervical carcinoma, as an experimental model. The action of ROSC was previously studied in detail in asynchronously growing [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2006, 2007c] and partially synchronized human MCF-7 breast cancer cells [David-Pfeuty, 1999] as well as in normal healthy cells [Alessi et al., 1998; Wesierska-Gadek et al., 2007a]. The susceptibility of asynchronously growing MCF-7 cells and cells released from a partial mitotic block substantially differed. Exposure of MCF-7 cells released from a partial mitotic block to ROSC slowed their G₁ to S progression [David-Pfeuty, 1999]. In contrast, ROSC arrested asynchronously growing MCF-7 cells in the G₂ phase of the cell cycle, and after longer exposure induced caspase-3 independent apoptosis. ROSC-induced cell death was mediated by wt p53. ROSC strongly enhanced the cellular expression of p53 protein and markedly extended its half-life [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Interestingly, ROSC-induced phosphorylation of p53 protein at serine 46 resulted in an up-regulation of p53AIP1 protein, its specific downstream target that after de novo synthesis and translocation into the mitochondria [Wesierska-Gadek et al., 2005a] initiated apoptosis in MCF-7 cells which lack caspase-3. Unlike in asynchronously growing MCF-7 cells, in those released from a partial mitotic block, ROSC slowed the transition of cells from G₁ to S phases [David-Pfeuty, 1999]. Surprisingly, reconstitution of MCF-7 cells with human caspase-3 did not sensitize them to the action of ROSC [Wesierska-Gadek et al., 2005a]. ROSC failed to induce P-Ser46 p53 protein and its downstream target in asynchronously growing, caspase-3 proficient MCF-7 cells [Wesierska-Gadek et al., 2005a], thereby indicating that

the outcomes of the inhibition of cellular CDKs strongly depends on cell types, their intrinsic features and cell cycle status prior to the onset of therapy. This observation was additionally substantiated by the fact that phenol red, a weakly estrogenic component of culture medium, affects the susceptibility of exponentially growing MCF-7 cells, that are known to express ER- α , to the action of ROSC [Wesierska-Gadek et al., 2006].

In the light of the above discussed cognitions it is incontrovertible that the outcomes of action of CDK inhibitors cannot be exactly predicted in different tumor cell lines, especially in those differing in the status of cell cycle and apoptosis regulators.

In this work we studied the effect of the CDK inhibitor ROSC on normal MRC-5 human fibroblasts and two human cervical carcinoma cell lines (HTB-31 and HeLa cells). HeLa cells are positive for HPV-18, while in HTB-31 no viral infection was detected. Both cancer cell lines differ in the p53 status. We determined the anti-proliferative and pro-apoptotic effect of ROSC on exponentially growing cells. Inhibition of CDKs most strongly affects the proliferation of HeLa cells. The marked reduction of the number of viable cells upon exposure of HeLa cells to ROSC is attributable to induction of cell cycle block at G₂/M and/or apoptosis. The outcome depends on the ROSC concentration. At higher dose ROSC eliminates HeLa cells by apoptosis. Moreover, ROSC represses the HPV-encoded oncoproteins. Unlike HeLa, HTB-31 cancer cells are markedly less susceptible to the tested CDK inhibitors. The ROSC-induced accumulation of the S- and G₂/M-phase population is not accompanied by apoptosis.

We also addressed the question whether the inhibition of CDKs in synchronized cells would be able to block the cell cycle in G₁ phase. For this purpose, we attempted to synchronize cells by serum withdrawal or by blocking the mitotic apparatus using nocodazole. Our results show that inhibition of CDKs in cells lacking the G₁/S checkpoint has different outcomes depending on the cell cycle status prior to the onset of treatment; ROSC does not inhibit HeLa cells in G₁-phase, but is solely able to maintain the early G₁-phase cell cycle arrest of cells released from the nocodazole-induced mitotic block. The results clearly show that ROSC effectively restricts growth of HeLa cells and the outcome is dose-dependent. It became evident that a higher ROSC concentration is necessary to promote apoptosis through abrogation of the activity of its inhibitors.

MATERIALS AND METHODS

CELLS

The human cervical carcinoma cell lines HeLaS₃, HTB-31 (C-33A), and normal MRC-5 human fibroblasts obtained from American Type Culture Collection (ATCC), were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS). Cells were grown up to 60–70% confluence and then treated with indicated drugs.

DRUGS

Roscovitin (ROSC) was prepared according to the published procedure [Havlicek et al., 1997] and prepared as 50 mM stock solution in DMSO. Aliquots of the stock solution were stored until use at –20°C. Nocodazole (NOC) from Sigma–Aldrich, Inc. (St. Louis,

MO) at a final concentration of 0.05 μ g/ml was used to induce mitotic block.

ANTIBODIES

The following specific antibodies were used to detect the relevant proteins: monoclonal anti-p53 antibody DO-1 (a kind gift from Dr. B. Vojtesek, Masaryk Memorial Cancer Institute, Czech Republic), the polyclonal anti-phospho-Thr161 CDK1, anti-phospho-Thr14/Tyr15 CDK1, anti-phospho-Thr160 CDK2, anti-phospho-Ser216 CDC25C, anti-phospho-Ser780 pRb, anti-phospho-Ser807/81 pRb, anti-phospho-Ser139-H2A.X, anti-phospho-Ser112 Bad, and corresponding antibodies against the total antigen (all from New England Biolabs, Beverly, MA), polyclonal anti-phospho-Ser164/Thr170 CDK7 and anti-phospho-Ser199 NPM (BioLegend, San Diego, CA), anti-caspase-3 (DAKO AS, Glostrup, Denmark), monoclonal anti-CDK2 (Ab-4) antibodies (Lab Vision Co., Fremont, CA), polyclonal-anti-phospho-Thr34 survivin, monoclonal anti-PCNA (clone PC-10), anti-pRb (IF-8), anti-cyclin A, anti-NPM (all from Santa Cruz Biotechnology, CA), anti-CDK7 (clone MO-1.1, Sigma–Aldrich, Inc.), anti-actin (clone C4, ICN Biochemicals, Aurora, OH), anti-E7 oncoprotein were from Abcam Ltd (Cambridge, England). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from R&D Systems (Minneapolis, MN).

DETERMINATION OF THE NUMBER OF VIABLE CELLS

Proliferation of human cervical carcinoma cell lines HeLaS₃, HTB-31 (C-33A), and normal MRC-5 human fibroblasts and their sensitivity to increasing concentrations of ROSC was determined by the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). As described recently in more detail [Wesierska-Gadek et al., 2005b], the CellTiter-Glo™ Luminescent Cell Viability Assay, generating luminescent signal, is based on quantification of the cellular ATP levels. Tests were performed at least in quadruplicates. Luminescence was measured in the Wallac 1420 Victor, a microplate luminescence reader. Each point represents the mean $\pm$ SD (bars) of replicates from at least three experiments.

DETERMINATION OF CASPASE-3/7 ACTIVITY

The activity of both caspases was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega Corporation) which uses the caspase-3/7 substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R100) as described previously [Wesierska-Gadek et al., 2005b]. Briefly, HeLaS₃ cells were plated in 96-well microtiter plates. One day after plating the cells were exposed for 24 h to increasing drug concentrations. Thereafter, the culture supernatant was transferred into another microtiter plate to separately determine the caspase activity in cells and in culture medium. Then an equal volume of caspase substrate was added and samples were incubated at 37°C for different periods of time to assess the best signal-to-background ratio. The fluorescence was measured at 485 nm. Luminescence and fluorescence were measured in the Wallac 1420 Victor, a microplate luminescence reader. Each point represents the mean $\pm$ SD (bars) of replicates from at least three experiments.

MEASUREMENT OF THE DNA CONTENT OF SINGLE CELLS BY FLOW CYTOMETRY

Measurement of the DNA-content was performed by flow cytometric analysis based on a slightly modified method [Wesierska-Gadek and Schmid, 2000] described previously by Vindelov et al. [1983]. The cells were detached from substratum by limited trypsinization, then all cells were harvested by centrifugation and washed in PBS. Aliquots of 10^6 cells were used for further analysis. Cells were stained with propidium iodide as described previously and then the fluorescence was measured using the Becton Dickinson FACScan after at least 2 h incubation at 4°C in the dark.

IMMUNOBLOTTING

Total cellular proteins dissolved in SDS sample buffer were separated on SDS slab gels, transferred electrophoretically onto PVDF membrane (PVDF) (Amersham Biosciences), and immunoblotted as previously described [Wojciechowski et al., 2003]. Equal protein loading was confirmed by Ponceau S staining. To determine the phosphorylation status of selected proteins, antibodies recognizing site-specifically phosphorylated proteins were diluted to a final concentration of 1:1,000 in 1% BSA in Tris-saline-Tween-20 buffer. In some cases, blots were used for sequential incubations.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software and significance levels were evaluated using Bonferroni's Multiple Comparison Test.

RESULTS

ROSC MORE STRONGLY INHIBITS THE PROLIFERATION OF HUMAN HeLa CERVICAL CANCER CELLS THAN THAT OF HTB-31 CELLS

To determine the effect of ROSC on the proliferation of exponentially growing human HeLa cells, the cells were continuously exposed to the drug for 12, 18, or 24 h, respectively. Then the cell number was determined using CellTiter-Glo viability assay immediately upon termination of the treatment, or alternatively the medium was changed, and then cells were post-incubated in a drug-free medium for a further 2 days and thereafter the assay was performed (Fig. 1A). According to the statistical analysis, 40 μ M ROSC very significantly reduced the number of living cells already after 12 h treatment. The effect was even stronger after longer incubation periods (18 and 24 h) and persisted upon medium change. Reduction of living cells following treatment with lower doses of ROSC (i.e., 10 and 20 μ M) was not statistically significant over the 24 h period. However, when cells incubated with 20 μ M ROSC for 24 h were transferred to the drug-free medium, the number of living cells was significantly reduced after the further 48 h cultivation.

In contrast, human HTB-31 cancer cells are less susceptible to the action of CDK inhibitors. Approximately a fourfold higher dose of ROSC is required to reduce the number of viable cells by 50% within 24 h (Fig. 1B).

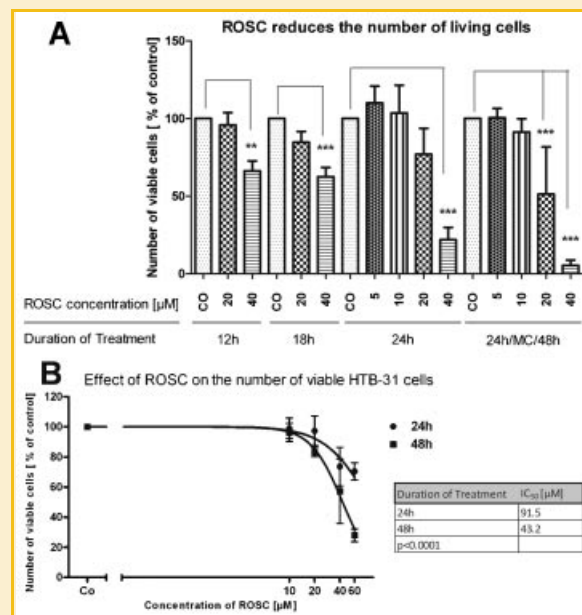


Fig. 1. Reduction of the number of viable human HeLa and HTB-31 cervical cancer cells after treatment with ROSC. A: Exponentially growing HeLa cells plated in 96-well microtiter plates were treated for 12, 18, and 24 h, respectively, with indicated concentrations of ROSC. The number of viable cells was determined directly after the treatment, or eventually after medium change (MC) and post-incubation for 48 h in a drug-free medium. The graph represents mean values from three independent experiments, each performed at least in quadruplicates. B: Exponentially growing HTB-31 cells plated in 96-well microtiter plates were treated for 24 and 48 h with ROSC. The differences between the number of control and treated cells are statistically highly significant (**; $0.001 < P < 0.01$) or very highly significant (*** $P < 0.001$) according to the Bonferroni's comparison.

As expected, normal human fibroblasts (MRC-5) were only negligibly affected by the selective CDK inhibitor. The IC₅₀ values are shown in Table I.

ROSC INHIBITS THE CELL CYCLE OF ASYNCHRONOUSLY GROWING HeLa CELLS AT THE G₂/M TRANSITION

The next experimental series were performed to find out how ROSC modulates the cell cycle progression and whether it is also able to induce apoptosis in HeLa cells. The DNA concentration in single cells was measured by flow cytometry. The population of hypoploid cells representing cells undergoing apoptotic changes was classified as a sub-G₁ population. HeLa cells were exposed to two concentrations of ROSC. As shown in Figure 2, ROSC at a final concentration of 20 μ M increased the frequency of G₂/M population and concomitantly

TABLE I. Treatment With ROSC for 24 h

Cell line	IC ₅₀ (μ M)
HeLa	28.1
HTB-31	91.5
MRC-5	Not achieved

diminished that of G₁-phase, with transient accumulation of S-phase after 18 h treatment. Two times higher concentration of ROSC increased the frequency of G₂/M population even more strongly at the same time point, but simultaneously, the population of hypoploid cells (30%) appeared after incubation for 18 and 24 h.

Concurrently, whole cell lysates obtained from HeLa cells treated with ROSC were analyzed by immunoblotting to monitor changes in

main cell cycle protein regulators (Fig. 3A,B). According to the predictions, phosphorylation of CDK2 at Thr160 was diminished in ROSC-treated cells indicating decreased activity of this kinase already after 12 h. The kinase responsible for activation of CDK2 by phosphorylating its Thr160 is CDK7, whose reduced phosphorylation at Ser164/Thr170 suggests one possible mechanism by which ROSC leads to inactivation of CDKs in cells. As expected,

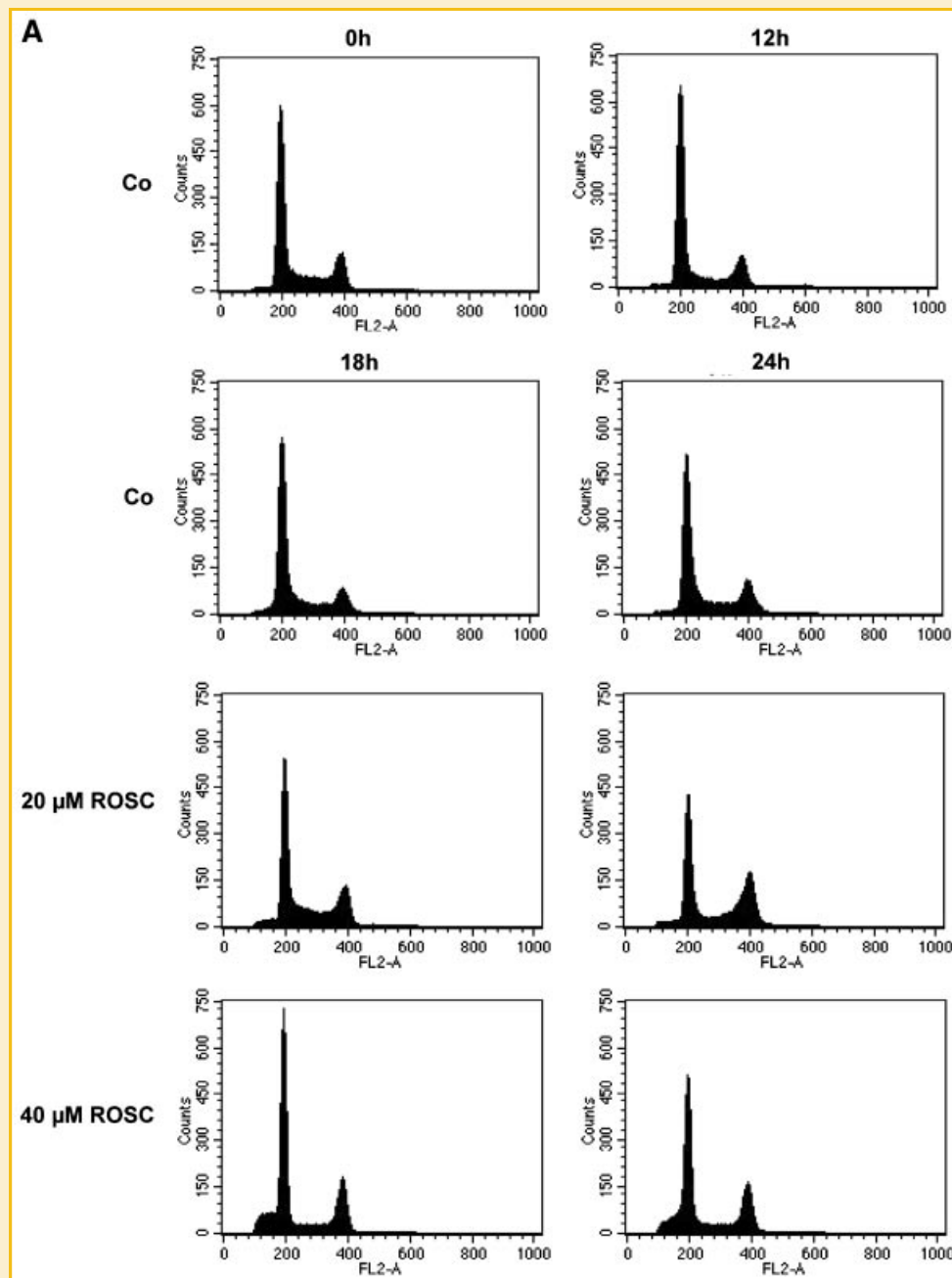


Fig. 2. Effect of ROSC on the cell cycle progression of asynchronously growing HeLa cells. Exponentially growing HeLa cells were treated with either 20 or 40 μ M ROSC for the indicated periods of time. Control and drug-treated cells were harvested by trypsinization and single cell suspension was used for propidium iodide staining. DNA content in single cells was measured using the Becton Dickinson FACScan flow cytometer. DNA histograms depicting a representative experiment performed in duplicate were prepared using the CellQuest evaluation program (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G₁ cell population were constructed (B). The distribution was determined using ModFIT cell cycle analysis software. The values represent a mean of two replicates.

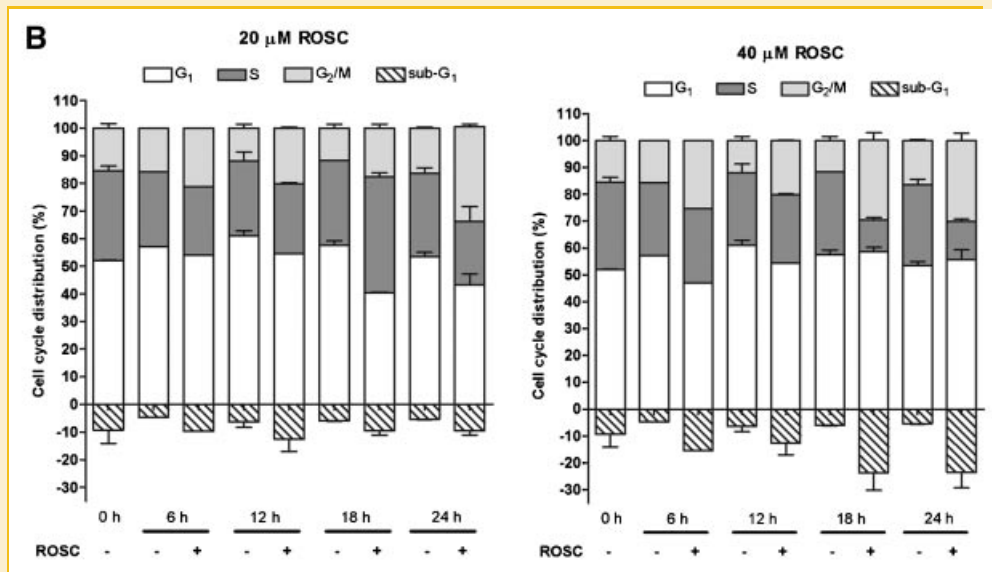


Fig. 2. (Continued)

ROSC prevented phosphorylation of pRb at Ser780 and Ser807/811, which correlates with decreased proliferation and observed reduction of G₁-phase in treated cells. However, pRb is phosphorylated by several CDKs, and one cannot discriminate which kinase is responsible for this effect. Therefore, we also analyzed phosphorylation of another CDK2-specific substrate—nucleophosmin (NPM) (Fig. 3B). A reduced signal from its phosphorylation at Thr199 is another evidence for inactivated CDK2. Surprisingly, after exposure of cells to 40 μ M ROSC for 24 h the site-specific phosphorylation of NPM increased. It remains to prove whether this event might be linked in any way to the ongoing apoptosis.

REPRESSION OF THE CELLULAR LEVELS OF HPV-ENCODED ONCOPROTEINS UPON ROSC TREATMENT

One might expect that inactivation of CDK7 and of cellular RNA polymerase II would shut down the expression of HPV-encoded proteins. Indeed, ROSC at high dosage reduced the levels of E6 (not shown) and E7 oncoproteins (Fig. 3C). The decrease of E7 protein was observed after treatment with ROSC for 18 h.

HIGHER DOSES OF ROSC INDUCE CASPASE-DEPENDENT APOPTOSIS IN HeLa CELLS

The exposure of HeLa cells to 40 μ M ROSC for 24 h resulted in a marked reduction of the number of viable cells. This effect seems to be attributable not only to the cell cycle arrest but additionally to the increased accumulation of a sub-G₁ cell population as detected by flow cytometry (Fig. 2), which is an indication of apoptosis. To substantiate this assumption, activity of caspase-3/7 in control and ROSC-treated cells was determined. The activity of cellular caspase-3/7 increased fourfold in HeLa cells treated with 40 μ M ROSC after 12 h or even more than eightfold after 24 h (Fig. 4A). Importantly, 20 μ M ROSC did not enhance activity of caspase-3/7 in HeLa cells even after 24 h. However, the long term effect of ROSC became evident

after medium change and post-incubation of ROSC-treated cells in a drug-free medium for a further 48 h. Activated effector caspases were released into the culture medium. An approximately hundredfold increase of caspase-3/7 activity in culture medium was detected with both doses of ROSC (Fig. 4B).

ROSC-MEDIATED ABROGATION OF PHOSPHORYLATION OF SURVIVIN AND OF BAD PRECEDES THE ONSET OF APOPTOSIS

To identify the mechanism by which inhibition of CDKs may contribute to the initiation of apoptosis, we examined the phosphorylation status of some factors regulating apoptosis. Interestingly, ROSC at the higher dose abolished site-specific phosphorylation of two proteins involved in the regulation of apoptosis: namely of survivin and of Bad (Fig. 5). The decrease of survivin phosphorylation starting after 18 h of exposure to 40 μ M but not to 20 μ M ROSC became even more evident after further 6 h. Beginning at 12 h treatment ROSC also clearly reduced and thereafter abolished phosphorylation of Bad at Ser112 rendering it disposed to heterodimerize with Bcl-2 proteins. ROSC-mediated decrease of site-specific phosphorylation of Bad did not affect its total level. Interestingly, this was accompanied by down-regulation of the tau form of 14-3-3 protein (Fig. 5). Considering the fact that after treatment with ROSC for 24 h CDK1 is inhibited by modification at Thr14/Tyr15 and (Fig. 3A) and CDC25C phosphatase is inactivated by phosphorylation at Ser216 (Fig. 5), one might speculate that coinciding abrogation of phosphorylation of surviving and Bad trigger apoptosis in G₂/M arrested cells.

The ROSC-mediated abrogation of the phosphorylation of both proteins seems to be a prerequisite for initiation of apoptosis in HeLa cells. Interestingly, exposure of more confluent HeLa cells to high ROSC dose failed to enhance the apoptosis and resulted in solely an accumulation of G₂/M arrested cells (results not shown). Remarkably, in this experimental series the phosphorylation of survivin and Bad was not abolished upon ROSC treatment even at the higher dose

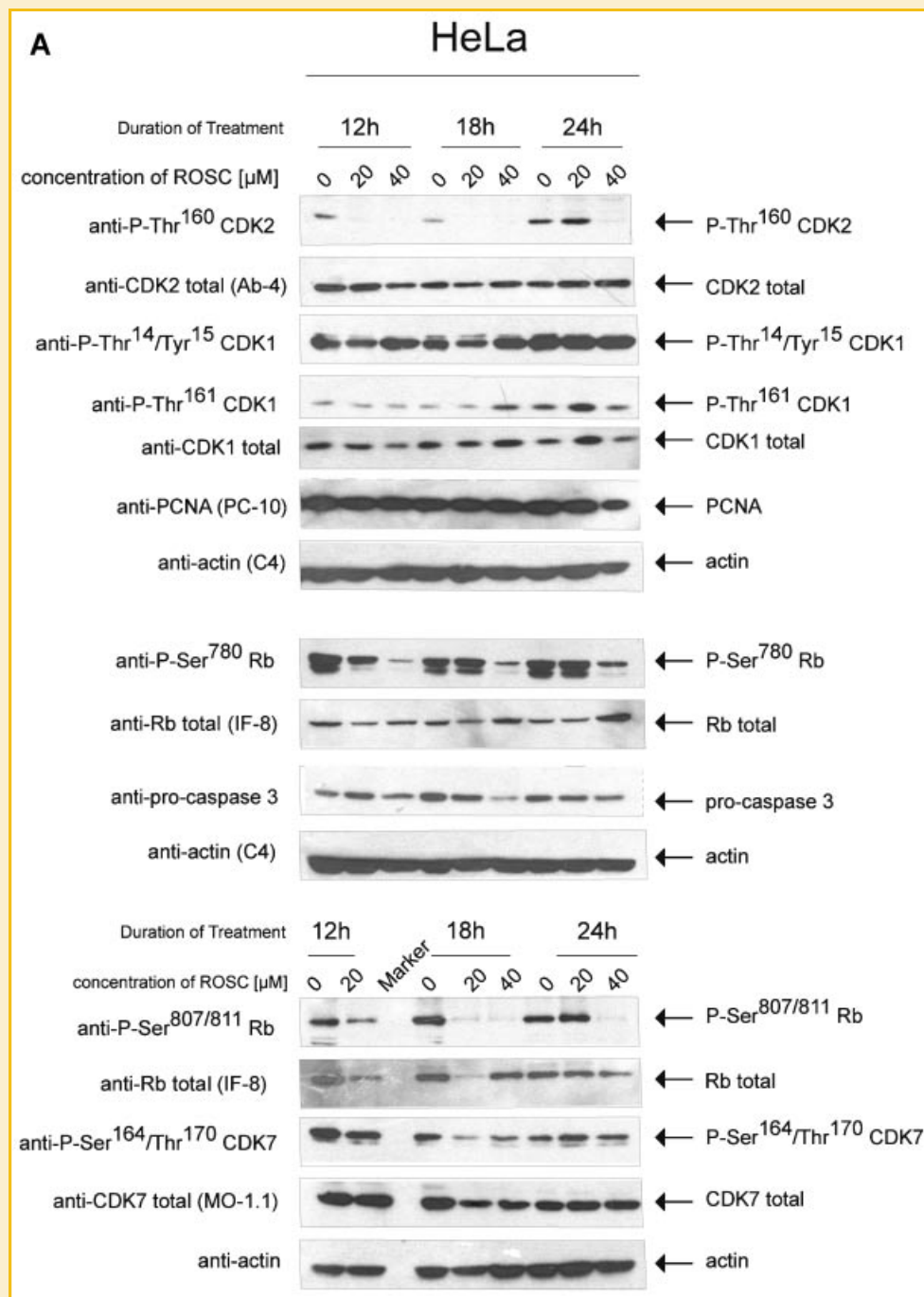


Fig. 3. Cell cycle regulators in asynchronously growing HeLa cells. Untreated and ROSC-treated HeLa cells were harvested and lysed. Whole cell lysates (WCLs) were loaded on 10% or 12% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

(results not shown), thereby indicating that their dephosphorylation is essential for initiation of cell death.

HTB-31 CELLS ACCUMULATE IN G₂/M BUT DO NOT DIE AFTER EXPOSURE TO ROSC

The exposure of HTB-31 cells to ROSC inhibits their cell cycle progression. As depicted in Figure 6A,B, HTB-31 cells accumulate in

the S- and G₂/M-phase. The frequency of the S-phase population of HTB-31 cells was differentially affected by both ROSC doses (Fig. 6B). At higher dosage percentage of S-phase cells was clearly diminished. After treatment with ROSC at a final concentration of 20 μ M frequency of G₁ cell population was clearly reduced. However, no substantial increase of the frequency of hypoploid cells was recorded (Fig. 6A,B).

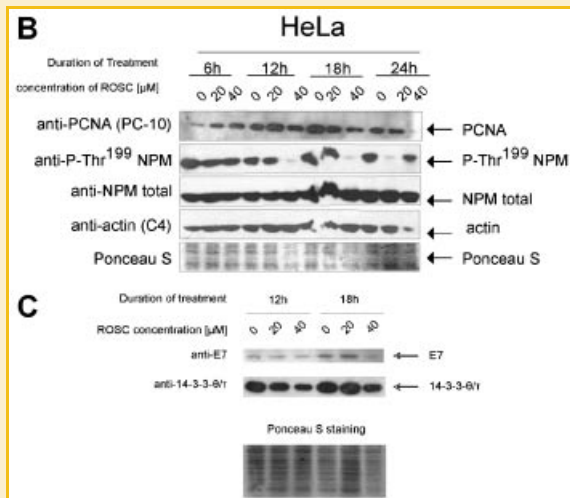


Fig. 3. (Continued)

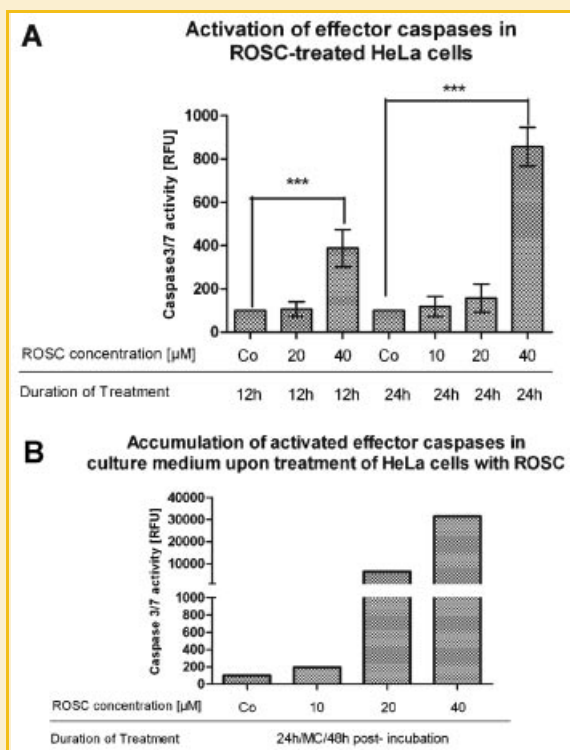


Fig. 4. Activation of caspase-3/7 in ROSC-treated HeLa cells. Exponentially growing HeLa cells were treated in a multiwell plate for either 12 or 24 h with ROSC at indicated concentrations. The activity of cellular caspase-3/7 (A) or caspase-3/7 released to the culture medium (B) was determined in quadruplicates using the APO-One Assay. The caspase-3/7 activity [relative fluorescence units (RFU)] $\pm$ SD was normalized to the number of viable cells that were determined by CellTiterGlo Assay. The differences between the caspase-3/7 activity in control and treated cells are statistically very highly significant ($***P < 0.001$) according to the Bonferroni's comparison.

ROSC DOES NOT ACTIVATE EFFECTOR CASPASES IN HTB-31 CELLS

Analysis of WCLs by immunoblotting revealed that levels of pro-caspase-3 in HTB-31 cells remain unchanged after ROSC treatment for 12 and 24 h (Fig. 6C) indicating that ROSC fails to activate caspase-3. This observation was additionally confirmed by determination of caspase-3/7 activity using the APO-One assay (not shown). Moreover, unlike in HeLa cells, in HTB-31 cells ROSC did not abolish the phosphorylation of survivin and of Bad. However, it is apparent that HTB-31 express lower levels of Bad protein (Fig. 6C) as compared to HeLa cells and other cancer cell lines. The lack of the activation of pro-apoptotic protein and effector caspases correlate with the results of measurement of DNA concentration in single HTB-31 cells performed by flow cytometry (Fig. 6A,B).

EFFECT OF SERUM WITHDRAWAL ON THE CELL CYCLE PROGRESSION OF HeLa CELLS

In the next experiment, the effect of serum withdrawal on the cell cycle progression in HeLa cells was examined. Unlike other cancer cell lines (e.g., MCF-7, used as a positive control), HeLa cells did not undergo G₁ block after serum deprivation when kept in a serum-free medium for 24 or 48 h (Fig. 7A,B). Surprisingly, serum starvation did not induce apoptosis in HeLa cells thereby confirming that the cells are able to survive and proliferate in the absence of growth factors.

Analysis of whole cell lysates obtained from serum-starved HeLa cells by immunoblotting revealed changes in major cell cycle regulators (Fig. 8). During starvation, a slow decrease of the activating phosphorylation of CDK2 and of the total protein level was observed (after 24 h) that was, however, recovered after serum supplementation. Interestingly, simultaneous increase in activating phosphorylation of CDK7 at Ser164/Thr170 was observed in starved cells, followed by its decrease in ROSC-treated cells. The level of site-specific phosphorylation of NPM, which was very low shortly after plating of HeLa cells, strongly increased after their cultivation for further 14 h (Fig. 8). Remarkably, it decreased after withdrawal of serum from the culture medium but not until 20 h and disappeared after serum supplementation (Fig. 8). The total level of nucleophosmin was less affected by these conditions

EFFECT OF ROSC ON HeLa CELLS RELEASED FROM SERUM STARVATION

Serum-starved HeLa cells were not able to arrest in G₁-phase even when sequentially treated with ROSC. A short ROSC treatment (for 12 h) of HeLa cells after re-feeding with serum for 4 h had no effect on the distribution of cells in the cell cycle phases. However, exposure of HeLa cells, serum starved for 24 h and then serum re-fed, to 40 μM ROSC for 24 h markedly reduced the population of G₁ cells and concomitantly led to accumulation of S- and G₂/M-phase cells (Fig. 7C). The distribution of HeLa cells in distinct cell cycle phases upon ROSC is reflected by the status of the main cell cycle regulators (Fig. 9). After a short treatment with ROSC (6 h), the drug reduced the site-specific phosphorylation and total level of CDK2 and CDK1, as compared to the cells after re-feeding for 10 h. This was accompanied by a slight decrease of the PCNA level. However, the increase of the ratio of S- and later of G₂/M-phase cells upon longer medication with ROSC coincides with the enhancement of activating phosphorylation of CDK2 and CDK1. Total levels of

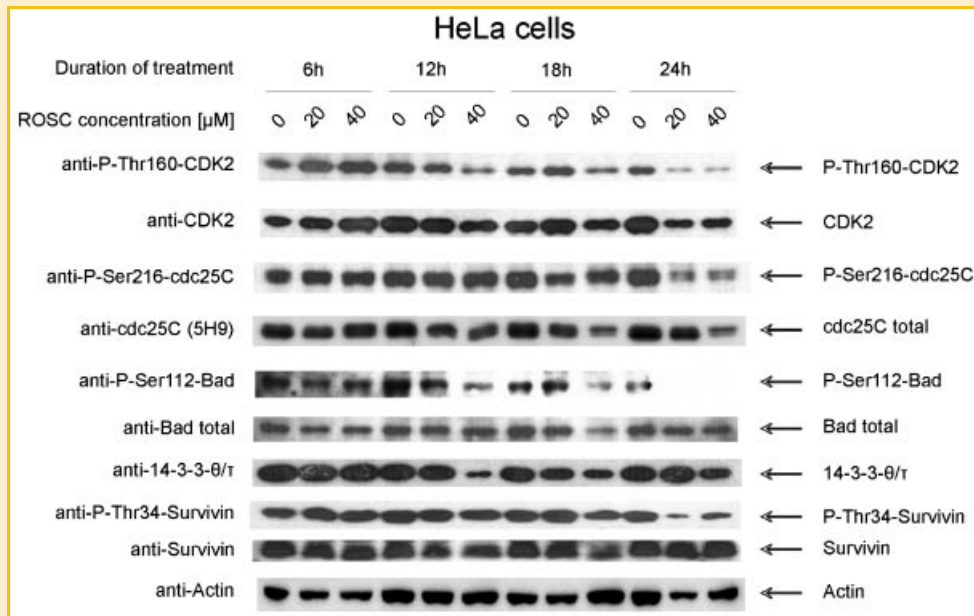


Fig. 5. ROSC-mediated abrogation of phosphorylation of survivin and of Bad in HeLa cells. Untreated control and ROSC-treated HeLa cells were harvested and lysed. Whole cell lysates (WCLs) were loaded on 10% or 12% SDS slab gels. Conditions of immunoblotting as described in detail in Figure 3.

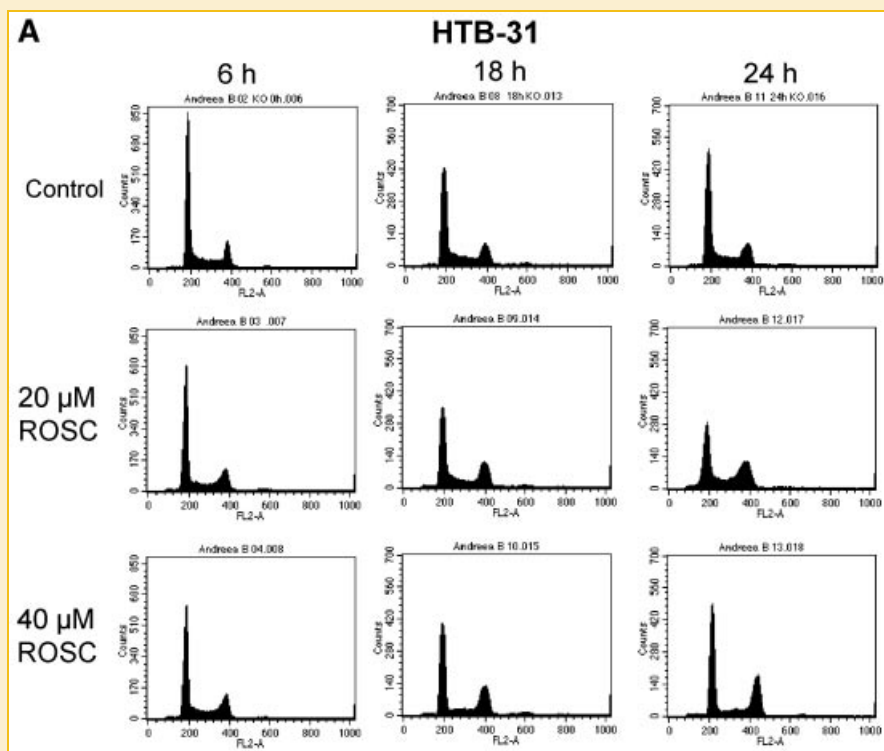


Fig. 6. Effect of ROSC on the cell cycle progression and cell cycle regulators in asynchronously growing HTB-31 cells. A,B: Cell cycle progression. Exponentially growing HTB-31 cells were treated with either 20 or 40 μ M ROSC for the indicated periods of time. Control and drug-treated cells were harvested by trypsinization and single cell suspension was used for propidium iodide staining. DNA histograms (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G₁ cell population (B). Conditions of staining and measurement as described in detail in the Figure 2. C: ROSC does not activate effector caspase-3 in HTB-31 cells. Untreated and ROSC-treated HTB-31 cells were harvested and lysed. Whole cell lysates (WCLs) were loaded on 12% or 15% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

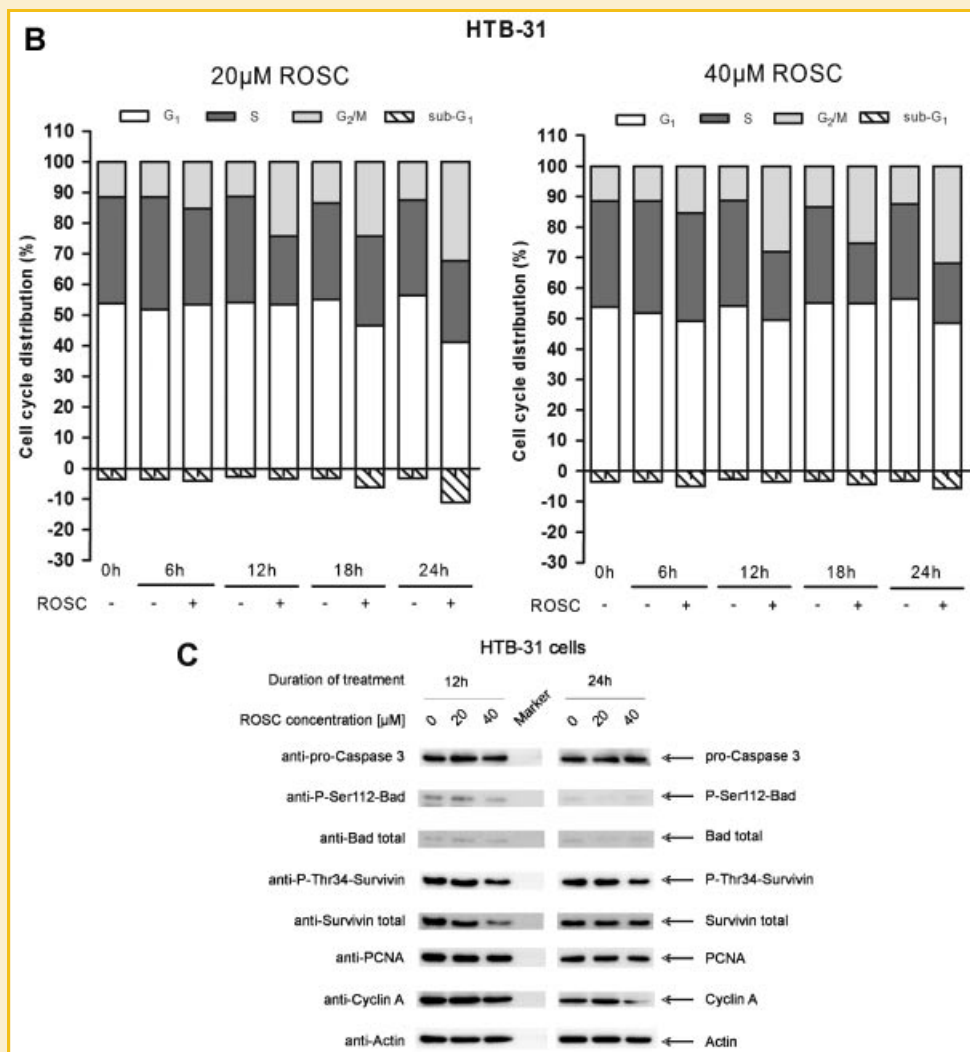


Fig. 6. (Continued)

CDK1 and CDK2 also increased (Fig. 9). Although the protein loading was not absolutely equal as evidenced by sequential incubation of the blots with anti-actin antibodies, the fluctuations of the intensity of the CDK1 and CDK2 bands are much stronger than those of actin and thereby support the results gained by flow cytometry.

ROSC MAINTAINS CELL CYCLE BLOCK IN CELLS ARRESTED IN EARLY G₁ PHASE

To verify the inability of ROSC to arrest serum-starved HeLa cells in G₁, we performed the cell cycle analysis of cells synchronized at G₂/M transition followed by a short release from the block and treatment with ROSC. Exposure of HeLa cells for 18 h to NOC at a final concentration of 0.05 μ g/ml resulted in a reversible mitotic block. Interestingly, when the cells were released from the block and progressed to early G₁ phase, they became much more susceptible to treatment with ROSC, preventing the cell cycle progression and resulting in a marked G₁ arrest (Fig. 10). The maintenance of G₁ arrest became clearly evident after treatment with ROSC for 12 h; HeLa cells accumulated in the G₁ phase even at lower concentration

of ROSC. However, the G₁ arrest was not permanent, the cell cycle progression was rather delayed and after 20 h treatment G₁ population decreased as cells passed through S phase to G₂/M. Progression through S to G₂/M monitored by flow cytometry was also supported by immunoblotting analysis of CDK1, showing not only accumulation of the total CDK1 protein level, but also its phosphorylation at Thr161 (Fig. 11). Similar changes were also observed with the CDK2 level and phosphorylation at Thr160. The DNA profile revealed that after release of HeLa cells from nocodazole-induced mitotic block the ratio of hypoploid cells markedly increased. To check whether after removal of the mitotic blocker DNA is damaged in cells entering the cell cycle, phosphorylation of histone H2A.X at Ser139 was determined. Indeed, after release of HeLa cells from the mitotic block for 4 h the site-specific phosphorylation of H2A.X increased and remained elevated during at least next 20 h in controls as well as ROSC treated cells (Fig. 11). ROSC also increased the cellular levels of p53 protein. However, the p53 increase in cells released from mitotic block was much weaker than that in asynchronously growing cells [Wesierska-Gadek et al., 2008a,b].

DISCUSSION

It is well documented that ROSC, a highly selective CDK inhibitor, influences cell cycle progression as a consequence of interference with multiple CDKs, but different outcomes are observed in different cell lines. Most often, cells arrest at G₂/M transition following ROSC application, as shown for example with asynchronously growing MCF-7 breast carcinoma, HCT-116 colon carcinoma, or K-562 chronic myelogenous leukaemia cell lines [Penuelas et al., 2003; Wojciechowski et al., 2003; Raynaud et al., 2005], despite weaker inhibition of the mitotic CDK1/cyclin B activity determined in vitro [McClue et al., 2002]. At the same time, however, an increased G₁ population and diminution of the number of S-phase cells was observed in MCF-7 cells or HT-29 colon carcinoma cells [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2006; Krystof et al., 2006], probably as a result of inhibition of CDK2 activity. Other CDK inhibitors were reported to have similar effects on the cell division cycle of animal cells; aminopurvalanol or indirubin-3'-monoxime strongly block G₂/M transition in the Chinese hamster CCL39 lung fibroblast cell line or in SV-40 transformed human HBL-100 breast epithelial cells, respectively [Damiens et al., 2001; Knockaert et al., 2002]. In synchronized cell populations, ROSC, as well as other CDK inhibitors, arrest the cell cycle progression depending on the cell cycle status prior to the onset of the treatment. MCF-7 cells partially synchronized by nocodazole in metaphase were primarily arrested in G₁ phase of the cell cycle after a release from the block and exposure to ROSC [David-Pfeuty, 1999] and the related compound aminopurvalanol induces a G₁ block in the serum-starved CCL39 cell line [Knockaert et al., 2002].

In this work, we studied the anti-proliferative and pro-apoptotic action of ROSC in two human HeLa cervical carcinoma cell lines: HeLa and HTB-31 cells. In HeLa cells the cell cycle is deregulated by infection with HPV-18 virus. Protein products of viral genes E6 and E7 deregulate the host cell growth cycle through binding and inactivating tumor suppressor proteins p53 and pRb, respectively. Considering this fact one might entertain suspicion that inhibition of CDK2 by ROSC would not affect cell cycle progression through G₁/S transition due to inactivation of pRb by E7 oncoprotein, our results were not so straightforward. ROSC indeed reduced the number of viable HeLa cells in the asynchronously growing culture by multiple mechanisms. At a lower dose, it rather directly blocked proliferation, as evidenced by accumulation of cells in G₂/M phases and concomitant decrease of G₁ population. However, at the double ROSC dosage, the frequency of G₁ population increased beginning from 12 h after onset of treatment. This outcome closely coincides with repression of virally encoded E6 [Wesierska-Gadek et al., 2008a] and E7 oncoproteins. The down-regulation of E6 viral product is essential for up-regulation and reactivation of p53 tumor suppressor protein [Wesierska-Gadek et al., 2008a,b], while the repression of E7 protein is crucial for abrogation of HPV-mediated disruption of the cell cycle control. Thus, it is a sign of the reconstitution of the G₁/S checkpoint in HeLa cells. The consequences of the ROSC-mediated effect became clearly evident during the monitoring of the phosphorylation status of pRb protein

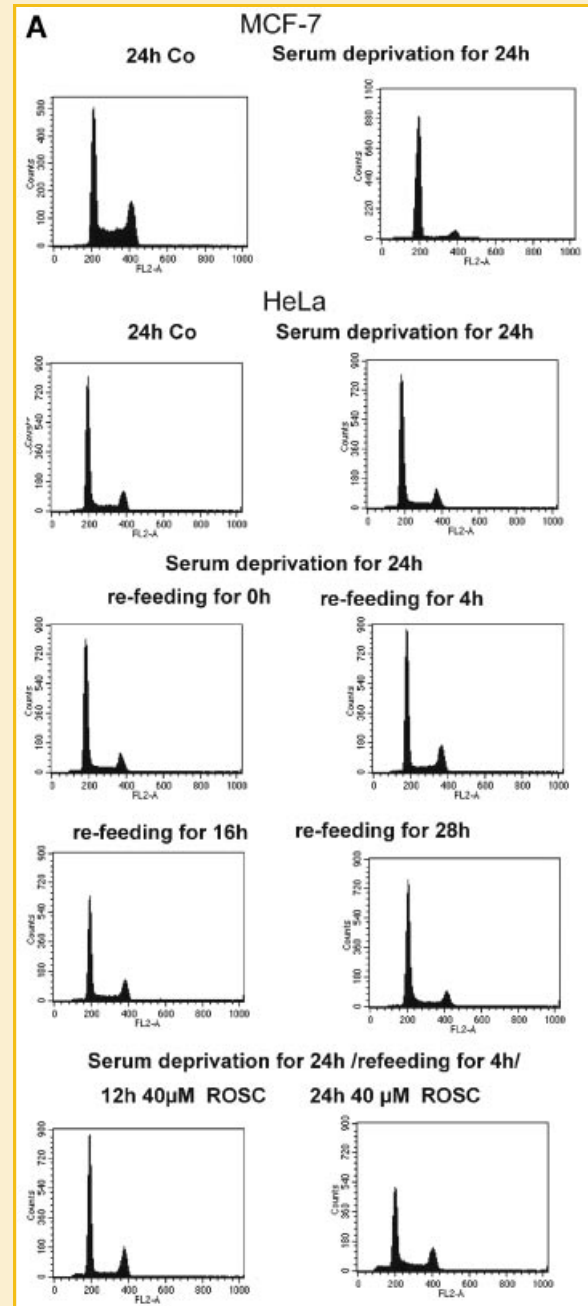


Fig. 7. Lack of G₁ synchronization of HeLa cells after serum deprivation. HeLa cells were plated under normal medium conditions. Twenty four hours after plating, medium was changed and cells were cultivated for indicated periods of time without FCS. At indicated time points cells were refed with serum and cultivated further for indicated periods in the absence or presence of ROSC. Then, cells were collected and prepared for measurement of DNA concentrations as described in more detail in Figure 2. MCF-7 cells were used as a positive control for serum starvation experiment. DNA histograms depicting a representative experiment were prepared using the CellQuest evaluation program (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G₁ cell population were constructed for cells kept without ROSC (B) or in the presence of ROSC (C).

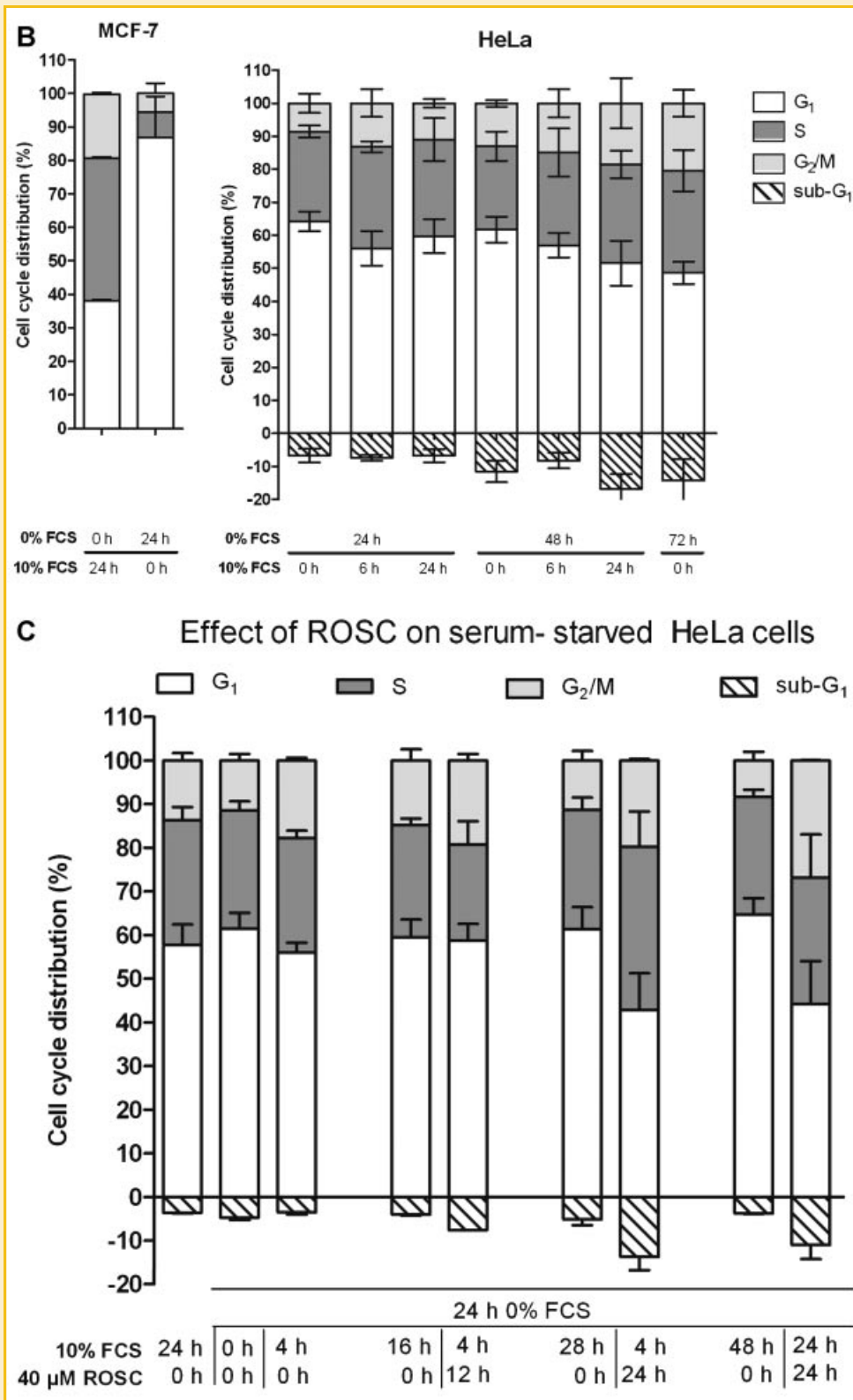


Fig. 7. (Continued)

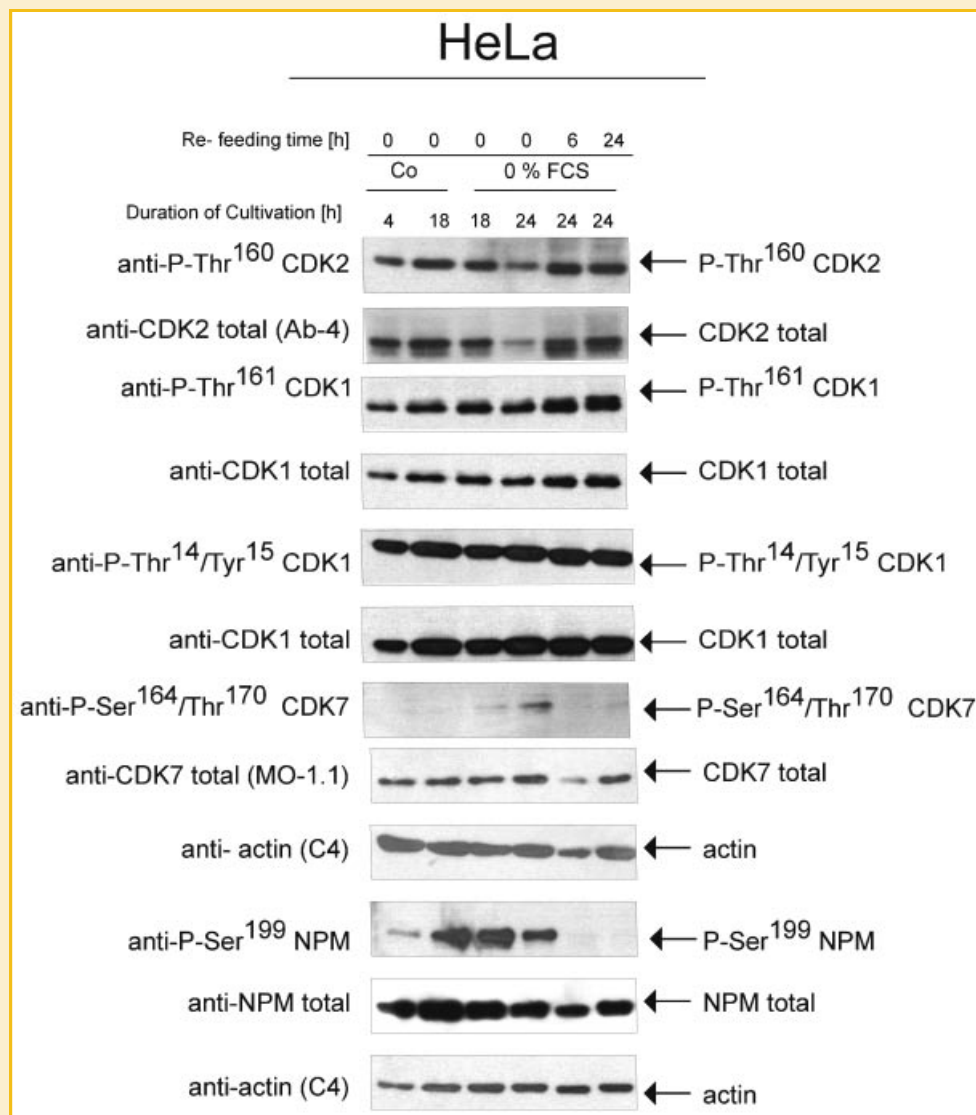


Fig. 8. Cell cycle regulators in HeLa cells after release from serum starvation. Control and serum-starved HeLa cells were harvested at the indicated time points and lysed. Whole cell lysates (WCLs) were loaded on 12% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

that showed decreased signal on immunoblots, as published earlier [Whittaker et al., 2004]. However, pRb is phosphorylated not only by CDK2, but also by CDK4, and therefore we analyzed phosphorylation of nucleophosmin (NPM) at Thr199 as another CDK2-specific substrate. Decreased phosphorylation of NPM following ROSC treatment confirmed inhibition of CDK2 in cells. By phosphorylating this particular residue at NPM, CDK2 allows the centrosome to be duplicated [Matsumoto et al., 1999; Okuda et al., 2000]. Thus, ROSC-mediated dephosphorylation of NPM is another mechanism by which this compound may exert its anti-proliferative action and directly contribute to the G2/M arrest.

ROSC not only limits cell cycle progression of HeLa cells, but after longer period of incubation, also eliminates the cells by induction of apoptosis, like in other cancer cell lines [Wojciechowski et al., 2003;

Wesierska-Gadek et al., 2005a, 2008b]. The ability of ROSC to induce caspase-dependent apoptosis is, however, manifested much more strongly after longer incubation periods and with higher doses of the inhibitor (Fig. 4). It has been shown that inactivation of single cell-cycle regulating CDK leads to cell cycle delay or arrest, while combined genetic or pharmacologic inactivation of CDK1, CDK2, CDK7, and CDK9 induces apoptosis through both E2F- and RNA polymerase II-mediated effects [Cai et al., 2006]. The action of ROSC in HeLa cells probably depends on its relative selectivity towards respective cellular targets, for example, CDK1, CDK2, CDK7, and CDK9 [McClue et al., 2002; Raynaud et al., 2005]. The effect of ROSC on the phosphorylation of CDK7 at Ser164/Thr170 has not been shown yet; it has been only known that ROSC directly inhibits the activity of CDK7 [Hajdich et al., 1999; McClue et al., 2002].

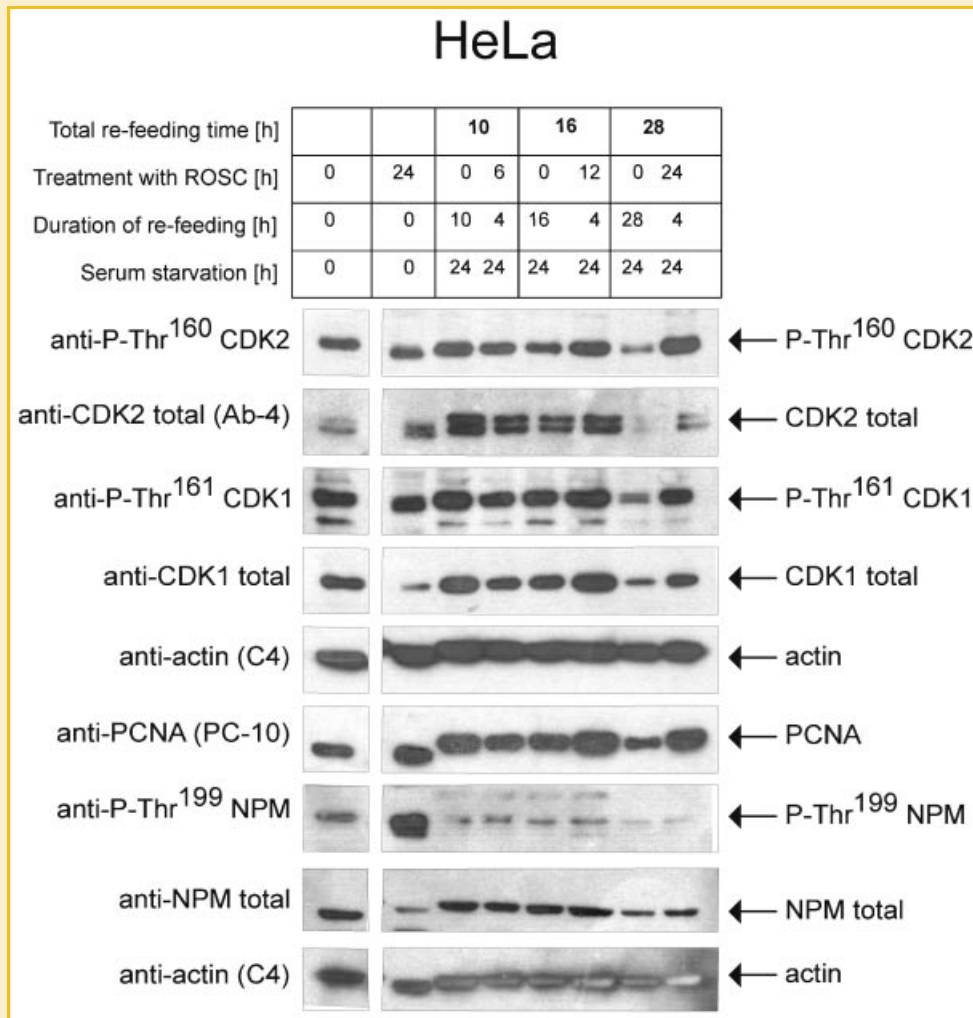


Fig. 9. Effect of ROSC on cell cycle regulators in HeLa cells after release from serum starvation. Untreated control and serum-starved HeLa cells were subsequently treated with ROSC for indicated periods of time, harvested and lysed. Whole cell lysates (WCLs) were loaded on 12% SDS slab gels. After electrophoretic transfer onto PVDF membrane, the proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by Ponceau S staining and by immunoblotting with anti-actin antibodies.

Similarly to other CDKs, where phosphorylated T-loop uncovers the catalytic site, its dephosphorylation leads to decreased CAK activity [Lolli et al., 2004], which was evidenced here by decreased phosphorylation of CDK1 and CDK2 on their T-loops (Figs. 3A and 8). Although it is not clear yet which kinase is responsible for activation of CDK7 by phosphorylating its T-loop, CDK7 can be activated by its targets CDK1 and CDK2 in vitro [Garrett et al., 2001; Lolli et al., 2004], which are ROSC-sensitive. Hence, activities of all CDK1, CDK2, and CDK7 may decrease in a feedback loop. The situation complicates functional redundancy of multiple CDKs; absence of one CDK is readily compensated with formation of unusual CDK/cyclin complexes as demonstrated by RNAi depletions of CDK1 and CDK2 [Cai et al., 2006]. Moreover, activity of CDK2 is apparently not absolutely dependent on CDK7, as it is able to activate itself by autophosphorylation at Thr160 [Abbas et al., 2007]. By inhibiting CDK1 and CDK2, ROSC in lower doses blocks cell cycle only, while its higher doses, that sufficiently inhibit more

kinases in cells (including CDK7), are able to initiate apoptosis, an outcome resembling depletion of multiple CDKs [Cai et al., 2006]. Our results clearly evidence the causal link between activity status of kinases and apoptosis in HeLa cells. We checked the site-specific phosphorylation of some inducers as well as inhibitors of apoptosis. The abrogation of the phosphorylation of two distinct regulators of apoptosis upon treatment with ROSC was achieved solely at higher ROSC dosage and preceded the onset of apoptosis. It is well established that the apoptosis promoting function of Bad protein strongly depends on its phosphorylation status [Harada et al., 1999]. Bad, a cytosolic protein is phosphorylated at serine residues 112 and 136 in response to growth factors resulting in its sequestration by the tau form of 14-3-3 protein. The complex formation with the tau form of 14-3-3 protein abrogates its heterodimerization with Bcl-xL, thereby promoting Bcl-xL-mediated cell survival. ROSC at higher concentration reduced and thereafter abolished Bad phosphorylation already after 12 h. It coincided with the decrease of the total

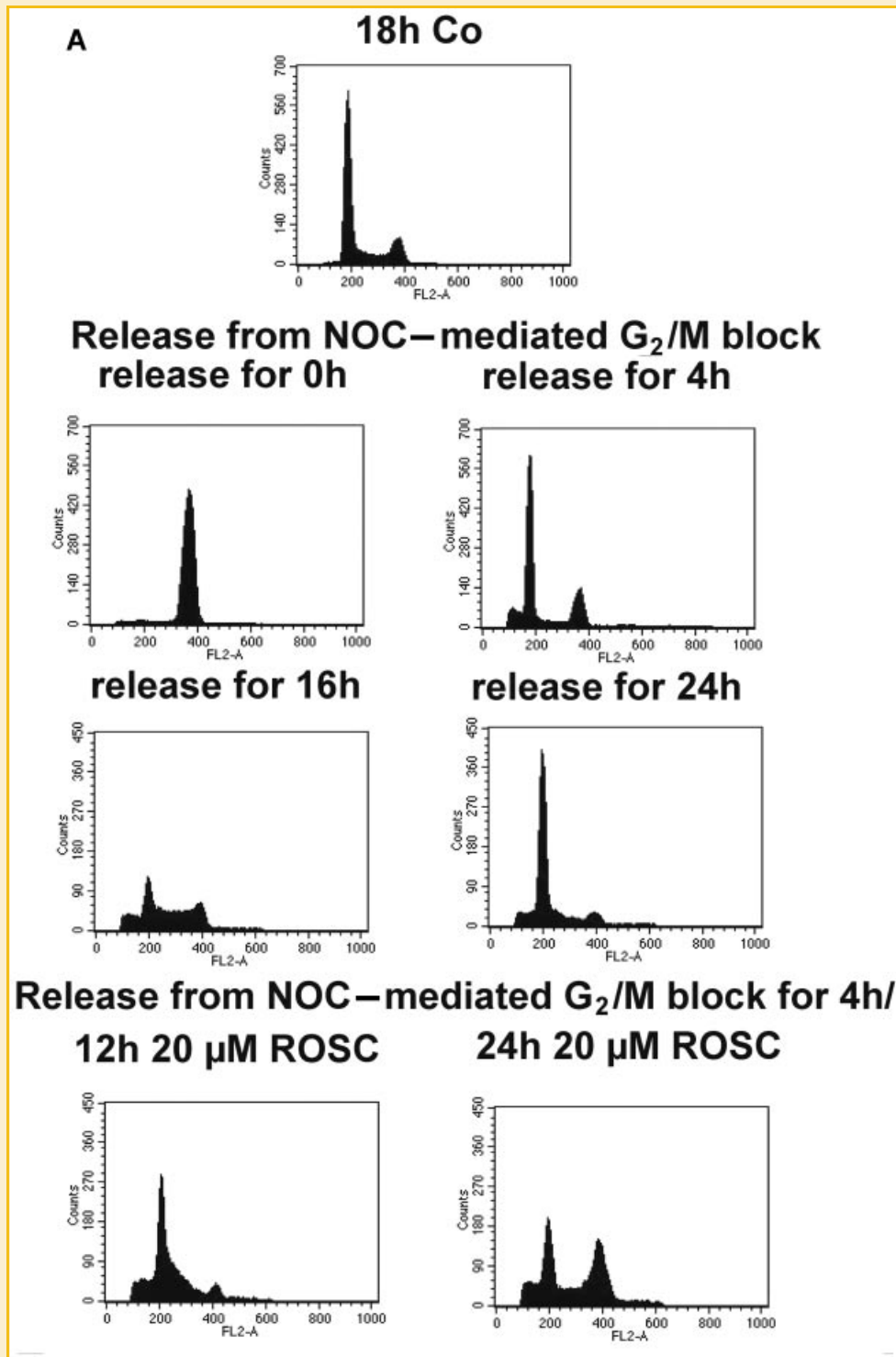


Fig. 10. Effect of ROSC on the cell cycle progression of HeLa cells synchronized by nocodazole. Exponentially growing HeLa cells were synchronized by treatment with nocodazole for 18 h. Then, cells were released from the G₂/M block by medium change and treated for indicated periods of time with either 20 or 40 μM ROSC. Then, cells were collected and prepared for measurement of DNA concentrations as described in more detail in Figure 2. DNA histograms depicting a representative experiment were prepared using the CellQuest evaluation program (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G₁ cell population were constructed (B).

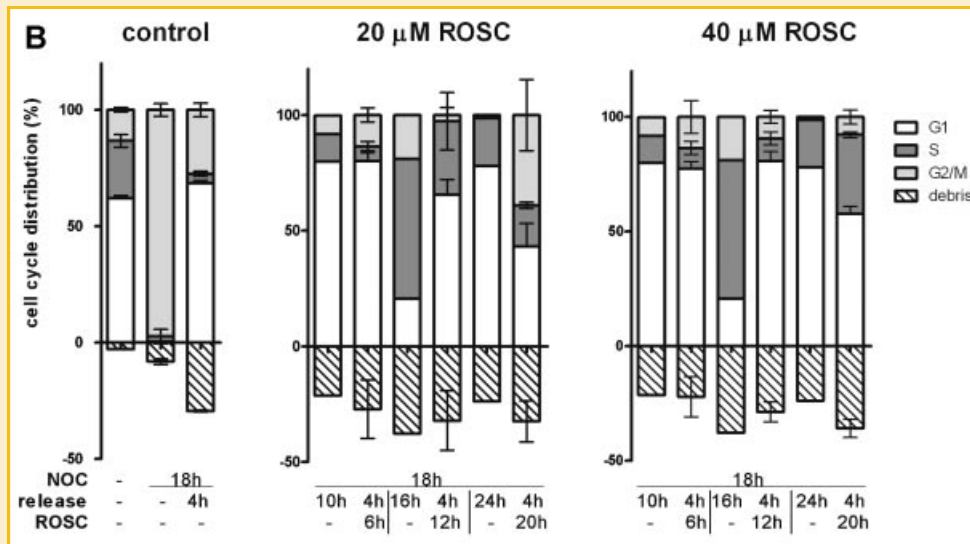


Fig. 10. (Continued)

level of 14-3-3 tau protein. ROSC also abrogated the phosphorylation of survivin, the smallest member of the family of inhibitors of apoptosis (IAPs). Survivin, a structurally unique IAP lacking a carboxy-terminal RING finger, is a bifunctional protein that acts not only as a suppressor of apoptosis, but is also an essential regulator of cell division [Altieri, 2003]. The highly increased expression of survivin in the majority of human malignancies, resulting in evasion of apoptosis and aberrant regulation of cell division was reported. Remarkably, survivin can be negatively regulated by functional p53 protein [Wesierska-Gadek and Schmid, 2007b] implicating that its expression and activity in response to ROSC treatment may be modulated by two independent mechanisms. The functional status and activity of survivin is regulated by phosphorylation [O'Connor et al., 2000]. Although survivin harbors a few phosphorylation sites that are known to be covalently modified by different cellular kinases, the phosphorylation of Thr34 catalyzed by CDK1 seems to be crucial for its anti-apoptotic activity [Wall et al., 2003]. Threonine 34 is ideally positioned in the acidic knuckle of the survivin molecule [Verdecia et al., 2000] to modulate its interaction with constituents of the apoptotic pathway such as caspase-9 or SMAC/DIABLO and to regulate the binding of client proteins controlling survivin stability. Phosphorylation of survivin at Thr34 increasing protein stability simultaneously enhances its interaction with caspase-9 and SMAC/DIABLO [Song et al., 2003]. Recently, multiple evidences were collected demonstrating that inhibition of survivin phosphorylation on Thr34 [Wall et al., 2003] or expression of non-phosphorylatable survivin mutant Thr³⁴ → Ala reduce its stability [Blanc-Brude et al., 2003]. It has been also reported that a single amino acid change within amino-terminus converts survivin activity from antiapoptotic to proapoptotic [Song et al., 2004]. These observations encouraged some oncologists to develop new therapeutic approaches based on the prevention of the survivin phosphorylation on Thr34 or its repression [Altieri, 2003; Lu et al., 2004; Zaffaroni et al. 2005].

The observed anti-proliferative and especially pro-apoptotic effect of ROSC is probably enhanced in a p53-dependent manner,

because this tumor suppressor accumulated upon longer incubation periods in HeLa cells [Wesierska-Gadek et al., 2008b]. The positive effect of ROSC on activation of p53 and induction of p53-dependent apoptosis has been described several times in the MCF-7 breast carcinoma cell line [David-Pfeuty, 1999; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Protein p53, as a transcriptional regulator, operates according to its post-translational modifications, especially phosphorylation and acetylation. For example when phosphorylated at Ser46, that is, at the residue important for its pro-apoptotic activity, p53 can transactivate a set of genes inducing apoptosis (e.g., *Bad*, *Puma*, *Noxa*, *p53AIP1*, *Bax*) and simultaneously suppress expression of anti-apoptotic genes (e.g., *survivin*, *bcl-2*) [Oda et al., 2000]. Moreover, p53 obviously induces apoptosis also independently of transcription; upon a stress signal, cytoplasmic p53 translocates to mitochondria, where it directly activates Bax, leading to the membrane depolarization and caspase activation [Chipuk et al., 2004]. Only recently we have found that ROSC is able to accumulate p53 phosphorylated at Ser46 also in HeLa cells, despite HPV infection [Wesierska-Gadek et al., 2008b]. Interestingly, accumulation of p53 in ROSC-treated HeLa cells is attributable to its stabilization by site-specific phosphorylation [Wesierska-Gadek et al., 2008b]. Moreover, the repression of cellular E6 oncoprotein levels following ROSC treatment allows p53 to restore its physiological activity [Wesierska-Gadek et al., 2008a].

As expected, serum starvation had no influence on progression of HeLa cells through G₁, and even ROSC, applied to the cells shortly after the release from starvation, was not able to maintain cells in G₁ phase, probably due to inactivation of the key G₁-regulator pRb by E7 oncoprotein. Although ROSC at high dosage down-regulates E7 oncoprotein, this effect takes place with some delay. Reduction of E7 in asynchronously growing HeLa cells was observed after 18 h. Conversely, populations in S-phase and G₂/M phases were significantly delayed following ROSC treatment for 24 h, probably as a consequence of unphosphorylated NPM that further block onset of mitosis [Matsumoto et al., 1999; Okuda et al., 2000]. In fact, the outcome is very similar to that in asynchronously growing cells,

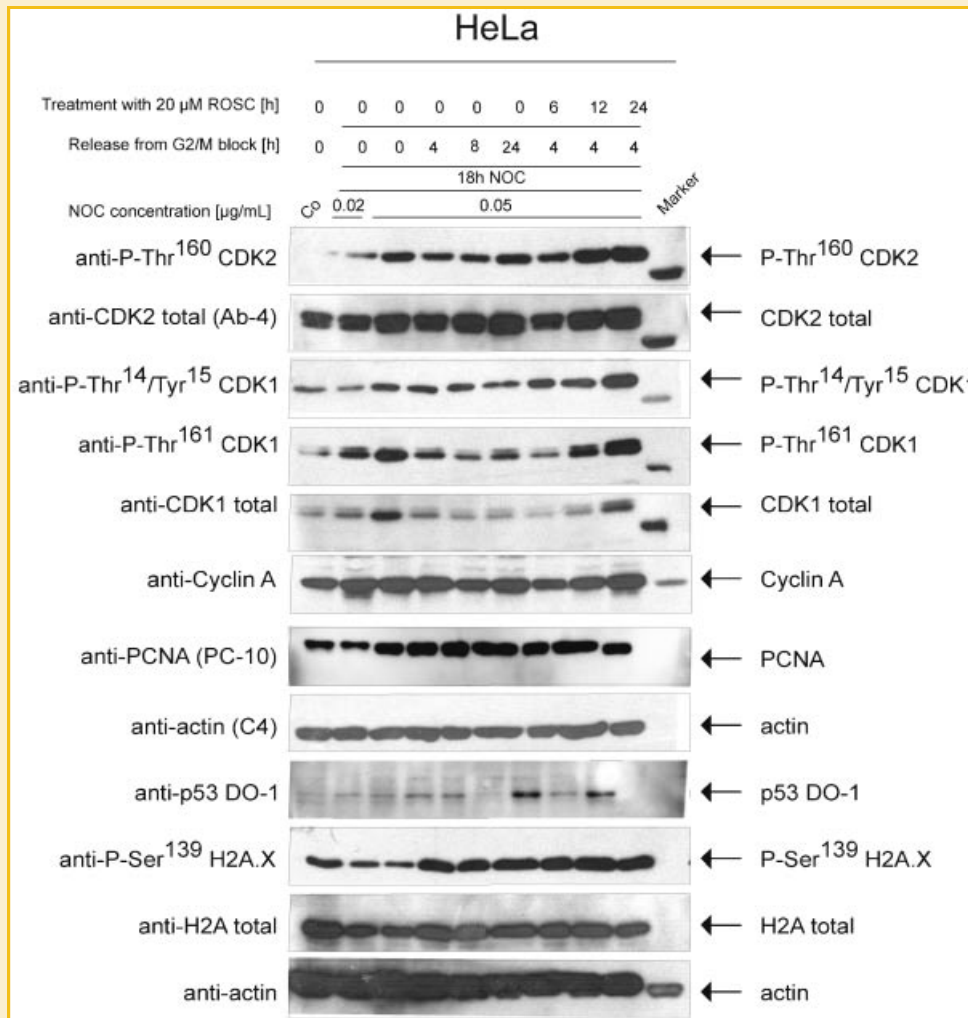


Fig. 11. Effect of ROSC on cell cycle regulators in NOC-synchronized HeLa cells. Unsynchronized control and NOC-synchronized HeLa cells were treated with ROSC for indicated periods of time, harvested and lysed. Whole cell lysates (WCLs) were loaded on 12 or 10% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

reflecting the heterogeneity of HeLa cells cultivated without the serum.

Although ROSC alone was not able to arrest HeLa cells in G₁ phase in either asynchronous or serum-starved culture, when released from nocodazole arrest, HeLa cells markedly delayed progression through G₁ phase when subsequently treated with ROSC (Fig. 10B). As the cells further proliferated and passed slowly through S phase to G₂, they also accumulated Thr-phosphorylated forms of CDK1 and CDK2 (Fig. 11).

In summary, ROSC has been shown to have different effects on HeLa cells depending on the concentration used and on the cell cycle status prior to the onset of treatment. Generally, lower doses reduce the proliferation rate by inhibition of cyclin-dependent kinases, while higher doses induce caspase-dependent apoptosis in HeLa cells. Moreover, the results also show that inhibition of CDKs by ROSC in cells lacking the G₁/S restriction checkpoint has different outcomes depending on the cell cycle status of the treated cells.

Considering the fact that after radiotherapy cancer cells are blocked at G₂/M, the ability of ROSC to induce apoptosis in G₂/M arrested cells may be of therapeutic importance.

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Impact of roscovitine, a selective CDK inhibitor, on cancer cells: bi-functionality increases its therapeutic potential[★]

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Increased expression and activity of proteins driving cell cycle progression as well as inactivation of endogenous inhibitors of cyclin-dependent kinases (CDKs) enhance the proliferative potential of cells. Escape of cells during malignant transformation from the proper cell cycle control rendering them independent from growth factors provides rationale for therapeutic targeting of CDKs. Exposure of rapidly growing human MCF-7 breast cancer and HeLa cervix cancer cells to roscovitine (ROSC), a selective inhibitor of CDKs, inhibits their proliferation by induction of cell cycle arrest and/or apoptosis. The outcome strongly depends on the intrinsic traits of the tumor cells, on their cell cycle status prior to the onset of treatment and also on ROSC concentration. At lower dose ROSC primarily inhibits the cell cycle-related CDKs resulting in a strong cell cycle arrest. Interestingly, ROSC arrests asynchronously growing cells at the G₂/M transition irrespective of the status of their restriction checkpoint. However, the exposure of cancer cells synchronized after serum starvation in the late G₁ phase results in a transient G₁ arrest only in cells displaying the intact G₁/S checkpoint. At higher dosage ROSC triggers apoptosis. In HeLa cells inhibition of the activity of CDK7 and, in consequence, that of RNA polymerase II is a major event that facilitates the initiation of caspase-dependent apoptosis. In contrast, in the caspase-3-deficient MCF-7 breast cancer cells ROSC induces apoptosis by a p53-dependent pathway. HIPK2-mediated activation of the p53 transcription factor by phosphorylation at Ser46 results in upregulation of p53AIP1 protein. This protein after *de novo* synthesis and translocation into the mitochondria promotes depolarization of the mitochondrial membrane.

Keywords: apoptosis, cell cycle arrest, cyclin-dependent kinases, inhibitors of cyclin-dependent kinases, roscovitine

INTRODUCTION

Proper progression of the cell division cycle is controlled in normal cells by activating and inhibiting cellular factors (for reviews, see Malumbres & Barbacid, 2005; Besson *et al.*, 2008). Each phase of the cell cycle is regulated by the complexes consisting of cyclin-dependent kinases (CDKs) and their

regulatory subunits called cyclins. Periodic activation of the catalytic constituent is, at least partially, attributable to the presence of their appropriate regulatory component. Unlike the kinases, cyclins fluctuate during the cell cycle. Their transient cell cycle-dependent expression is regulated by both *de novo* synthesis and by proteasome mediated degradation. Apart from the activating factors, the cell

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Abbreviations: CAK, cyclin-dependent kinase-activating kinase; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; CTD, carboxy-terminal domain of RNA polymerase II; HIPK2, homeodomain-interacting protein kinase-2; HPV, human papilloma virus; INK4, inhibitor of CDK4; p53AIP1, p53-regulated apoptosis-inducing protein 1; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; pRb, retinoblastoma protein; RIPA, radioimmunoprecipitation assay; ROSC, roscovitine; WCLs; whole cell lysates; wt, wild type.

cycle is additionally regulated by inhibitory factors like inhibitors of CDKs (CKI) or tumor suppressor proteins (e.g., p53, pRb or Pten). The fine-tuned balance between these positive- and negative-acting regulators guarantees proper cell cycle progression in normal cells and facilitates adequate cellular responses to different physiological conditions like exhaustion of nutrients and a variety of stress stimuli. A constitutive activation or increased expression of the positive regulators of the cell cycle in cancer cells is frequently accompanied by a loss or inactivation of inhibitors of CDKs (Malumbres & Barbacid, 2007). The deregulation of the inhibitors of CDKs commonly observed in many types of human malignancies is attributable to the deletion or silencing of genes (e.g., products of the *INK4A* gene) or to impaired protein synthesis, mislocalization or enhanced protein destruction (e.g., p27^{Kip1} protein). Degradation of such cell cycle regulators is carried out by the ubiquitin system in which covalent attachment of polyubiquitin targets proteins for proteolysis by the proteasome. Although the specificity of the polyubiquitylation system is mainly determined by the ubiquitin ligase complex, the recognition of substrates seems to be mediated by another mechanism. It has been demonstrated that the stability of p27^{Kip1} is regulated by threonine phosphorylation in position 187. Only phosphorylated p27^{Kip1} molecules are targeted by Skp2, a component of the SCF ubiquitin ligase complex. The critical modification of p27^{Kip1} protein is probably mediated by the CDK2-cyclin E complex (Besson *et al.*, 2008). Thus, CDKs seem to control distinct events in the cell cycle progression at multiple levels. The fact that cancer cells escape from the cell control cycle and acquire unlimited proliferation potential as a consequence of a loss or inactivation of endogenous CKIs provides rationale to counteract this deficit by administration of pharmacological inhibitors of CDKS for cancer therapy (Fischer & Lane, 2005; Malumbres *et al.*, 2008).

In this paper the action of roscovitine (ROSC), a CDK inhibitors belonging to trisubstituted purines, on human cancer cells differing in the status of the restriction checkpoint was examined. Asynchronously growing human MCF-7 breast cancer, and HeLa cervical cancer cells were exposed to ROSC. Inhibition of CDKs arrested the cancer cells in G₂/M and induced apoptosis. The kinetics of cell cycle arrest and concentration dependency differed between the two cell lines. Moreover, the CDK inhibitor triggered apoptosis by activation of caspase-dependent or independent pathways. ROSC simultaneously inhibited cell cycle progression and transcriptional elongation. The latter had a strong impact on virally encoded oncoproteins. Interestingly, ROSC showed a weak inhibitory effect on proliferation and cell cy-

cle progression of normal human MRC-5 and F2000 fibroblasts (Węsierska-Gądek *et al.*, 2008a).

Our results clearly evidence that the bi-functionality of some pharmacological inhibitors of CDKS like ROSC markedly enhances their therapeutic efficacy *via* targeting several cellular pathways.

MATERIALS AND METHODS

Cells. The human MCF-7 breast cancer and HeLaS₃ cervical carcinoma cell lines obtained from American Type Culture Collection (ATCC), were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS). Cells were grown up to 60% confluence and then treated with indicated drugs.

Drugs. Roscovitine (ROSC) was prepared according to the published procedure (Havlicek *et al.*, 1997) and prepared as a 50 mM stock solution in DMSO (dimethylsulfoxide). Aliquots of the stock solution were stored until use at -20°C.

Cell treatment. Cells were treated with ROSC at a final concentration ranging from 1 to 60 µM for indicated periods of time. The highest concentration of solvent (DMSO) did not exceed 1%. DMSO at this concentration had no detectable effect on cell cycle progression and on the number of living cells.

Antibodies. The following specific antibodies were used to detect the relevant proteins: monoclonal anti-p53 antibody DO-1 (a kind gift from Dr. B. Vojtesek, Masaryk Memorial Cancer Institute, Czech Republic), polyclonal anti-phospho-Ser46 p53, anti-phospho-Thr160 CDK2 and anti-phospho-Ser780 pRb (all from New England Biolabs, Beverly, MA, USA), polyclonal anti-phospho-Ser164/Thr170 CDK7 and monoclonal anti-MCM-7 antibodies (clone DCS-141) (BioLegend, San Diego, CA, USA), monoclonal anti-CDK2 (Clone 2B6 +8D4) antibodies (Lab Vision Co., Fremont, CA), monoclonal anti-PCNA (clone PC-10), anti-pRb (IF-8), (all from Santa Cruz Biotechnology, CA, USA), anti-CDK7 (clone MO-1.1, Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal (BF7) antibodies to HPV18 E6, mouse monoclonal antibodies (H5) to phospho-Ser2 RNA polymerase II, mouse monoclonal antibodies (H14) to phospho-Ser5 RNA polymerase II (Abcam Cambridge, UK), monoclonal antibodies to RNA polymerase II (clone ARNA-3), (Acris Antibodies GmbH, Herford, Germany), and anti-actin (clone C4, ICN Biochemicals, Aurora, OH, USA). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from R&D Systems (Minneapolis, MN, USA).

Determination of the number of viable cells. Proliferation of human MCF-7 breast cancer and HeLaS₃ cervical carcinoma cells and their sensitivity to increasing concentrations of ROSC was determined by the CellTiter-Glo™ Luminescent Cell

Viability Assay (Promega Corporation, Madison, WI, USA). As described recently in more detail (Wesierska-Gadek *et al.*, 2007), the assay, generating a luminescent signal, is based on quantification of cellular ATP. Tests were performed at least in quadruplicates. Luminescence was measured in a Wallac 1420 Victor microplate luminescence reader. Each point represents the mean $\pm$ S.D. (bars) for at least four experiments.

Measurement of DNA content of single cells by flow cytometry. Measurement of the DNA content was performed by flow cytometric analysis based on a slightly modified method (Wesierska-Gadek & Schmid, 2000) described previously by Vindelov *et al.* (1983). The cells were detached from substratum by limited trypsinization, harvested by centrifugation and washed in PBS (phosphate-buffered saline). Aliquots of 10^6 cells were used for further analysis. Cells were stained with propidium iodide as described previously and then the fluorescence was measured using a Becton Dickinson FACScan after at least 2 h incubation at 4°C in the dark.

Immunoblotting. Total cellular proteins dissolved in SDS sample buffer were separated on SDS/polyacrylamide slab gels, transferred electrophoretically onto polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences), and immunoblotted as previously described (Wojciechowski *et al.*, 2003). Equal protein loading was confirmed by Ponceau S staining. To avoid non-specific protein binding sites, the membranes were saturated with 5% non-fat dry milk in TBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl) for at least 1 h at room temperature. After extensive washing in TBS containing 0.05% Tween 20 (TBST), blots were incubated with specific primary antibodies at an appropriate final dilution and the immune complexes were detected using appropri-

ate HRP-conjugated secondary antibodies and the enhanced chemiluminescent detection reagent ECL+ (Amersham International, Little Chalfont, Buckinghamshire, England) (Wesierska-Gadek *et al.*, 2005). To determine the phosphorylation status of selected proteins, antibodies recognizing site-specifically phosphorylated proteins were diluted to a final concentration of 1:1000 in 1% BSA (bovine serum albumin) in Tris/saline/Tween-20 buffer. In some cases, blots were used for sequential incubations. Chemiluminescence was analysed and documented using ChemiSmart 5100 equipped with a high resolution camera and image master software. Acquisition of chemiluminescence images using ChemiSmart 5100 offers unrivalled sensitivity and maximum dynamic range. Incubation with anti-actin antibodies confirmed equal protein loading.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software and significance levels were evaluated using Bonferroni's Multiple Comparison Test.

RESULTS

ROSC more strongly inhibits proliferation of human MCF-7 breast cancer cells than that of HeLa cervical cancer cells

To determine the anti-proliferative effect of ROSC on exponentially growing human cancer cells differing in the G_1/S checkpoint status (HeLa and MCF-7 cells), the cells were continuously exposed to the drug for 24 h. Then the cell number was determined using the CellTiterLumiGlo viability assay immediately upon termination of the treatment, or alternatively the medium was changed and the cells

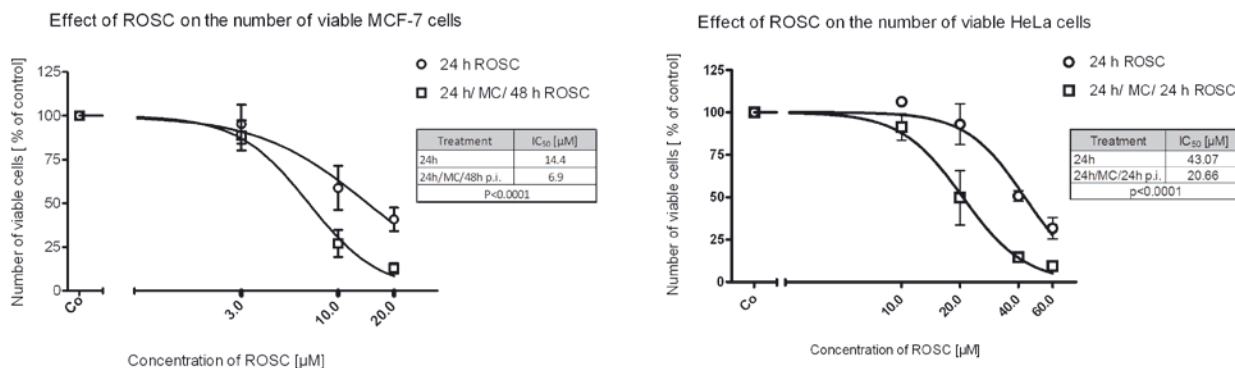


Figure 1. Reduction of the number of viable human MCF-7 breast cancer and HeLa cervical cancer cells after treatment with ROSC.

Exponentially growing cells plated in 96-well microtiter plates were treated for 24 h with indicated concentrations of ROSC. The number of viable cells was determined directly after the treatment, or additionally after medium change (MC) and post-incubation for 48 h (MCF-7 cells, left panel) or for 24 h (HeLa cells, right panel) in a drug-free medium. The graph represents mean values from three independent experiments, each performed at least in quadruplicates. The differences between the number of control and treated cells are statistically highly significant ($^{**}0.001 < P < 0.01$) or very highly significant ($^{***}P < 0.001$) according to the Bonferroni's comparison.

were post-incubated in a drug-free medium for a further 1 or 2 days and the assay was performed then (Fig. 1). ROSC affected the proliferation of MCF-7 cells more strongly than that of HeLa cells. An approximately three-fold higher drug concentration was necessary to reduce the number of living HeLa cells by 50%. Remarkably, when cells incubated with ROSC for 24 h were transferred to drug-free medium, the number of living cells was significantly reduced further after cultivation for 24 h (HeLa cells) or 48 h (MCF-7 cells). The IC_{50} values decreased approximately three-fold as shown in Fig. 1. These results evidence that the effect of ROSC on cancer cells is prolonged and even during post-incubation in a drug-free medium a delayed outcome becomes apparent.

ROSC inhibits the cell cycle of asynchronously growing MCF-7 and HeLa cells at the G_2/M transition with different kinetics

The next experimental series was performed to find out how ROSC modulates the cell cycle progression of the cancer cells tested and whether it is also able to induce apoptosis in HeLa cells. The DNA concentration in single cells was measured by flow cytometry. The population of hypoploid cells representing cells undergoing apoptotic changes was classified as a sub- G_1 population. Interestingly, MCF-7 cells rapidly responded to 20 μ M ROSC, whereas in HeLa cells the changes in the distribution of cell cycle phases became evident after 24 h (Figs. 2 and 3). In MCF-7 cells ROSC at a final concentration of 20 μ M increased the frequency of the G_2/M population already after 8 h and concomitantly diminished that of G_1 - and S-phase. At this dosage ROSC exerted a much weaker and delayed effect on HeLa cells (Figs. 2 and 3). These results are in concordance with the data provided by cell viability tests (Fig. 1). Further experiments revealed that a two-fold higher concentration of ROSC increased the frequency of G_2/M population of HeLa cells more strongly at the same time point, and simultaneously, the population of hypoploid cells (30%) appeared after incubation for 18 h and 24 h (not shown). In contrast, after exposure of MCF-7 cells to ROSC no hypoploid cells were detected (Figs. 2 and 3). This observation is not surprising because these cells are apoptosis-resistant due to disruption of the gene encoding caspase-3.

ROSC inhibits global transcription resulting in repression of virally-encoded oncoproteins

The lower susceptibility of human HeLa cervical cancer cells to the inhibition of cellular CDKs is not surprising since they are HPV-18-positive. The virally encoded E6 und E7 oncoproteins inacti-

vate wt p53 tumor suppressor protein and the G_1/S checkpoint, respectively (Scheffner *et al.*, 1990; Helt & Galoway 2003). Therefore, we decided to assess the effect of ROSC on the expression of HPV-encoded proteins. Whole cell lysates (WCLs) were prepared from ROSC-treated HeLa cells concurrently to the flow cytometric measurement, and were subsequently analysed by immunoblotting to monitor changes in major cell cycle protein regulators (Fig. 4). ROSC inhibited in a time- and concentration-dependent

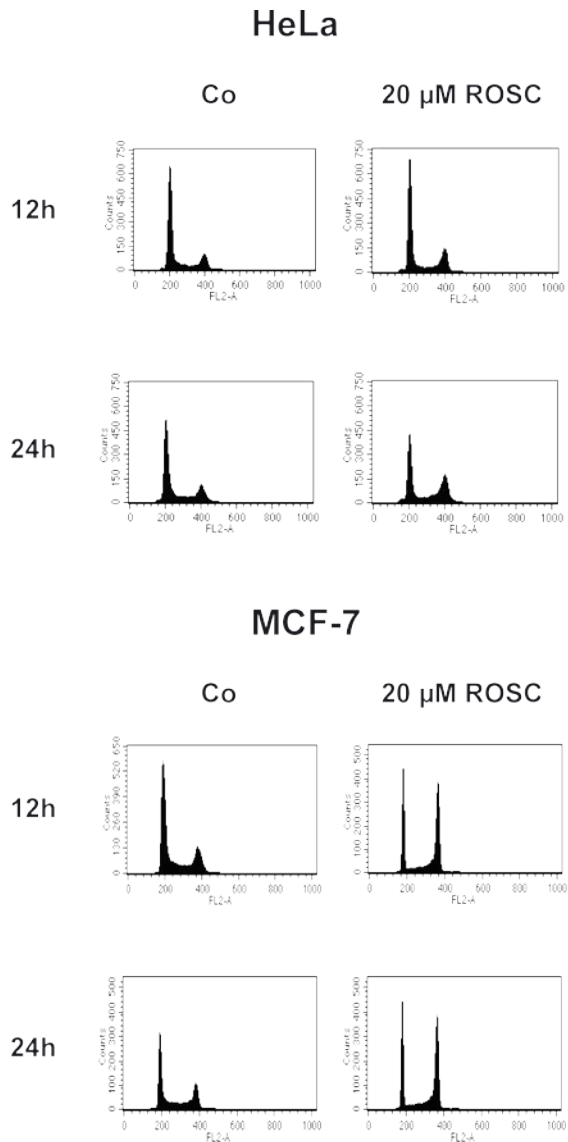


Figure 2. Effect of ROSC on cell cycle progression of asynchronously growing human MCF-7 and HeLa cells.

Exponentially growing MCF-7 and HeLa cells were treated with 20 μ M ROSC for the indicated periods of time. Control and drug-treated cells were harvested by trypsinization and single-cell suspension was used for propidium iodide staining. DNA content in single cells was measured using Becton Dickinson FACScan flow cytometer. DNA histograms depicting a representative experiment performed in duplicate were prepared using the CellQuest evaluation program.

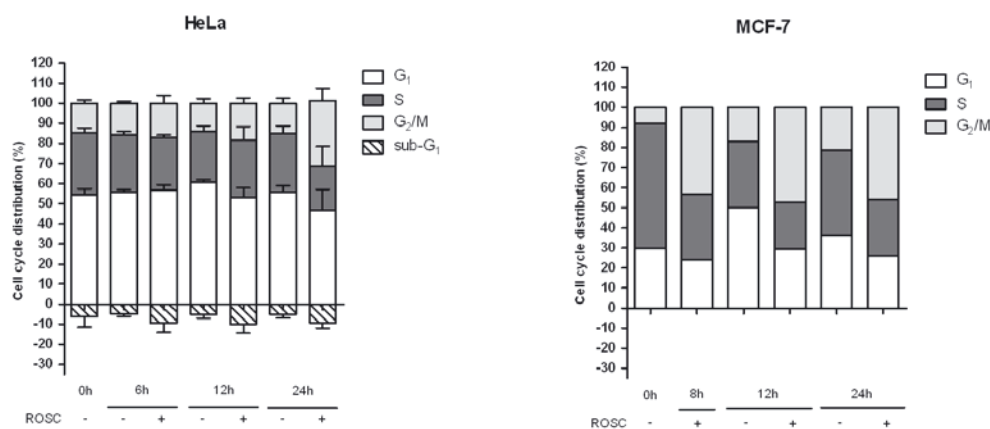


Figure 3. Rapid G₂/M arrest of human MCF-7 breast cancer cells after exposure to ROSC.

Diagrams show changed distribution of HeLa and MCF-7 cells in distinct cell cycle phases and the frequency of sub-G₁ cell population. The distribution was determined using ModFIT cell cycle analysis software. Values for HeLa cells represent a mean of two replicates.

manner the activating phosphorylation of CDK7 and, in consequence, that of RNA polymerase II (Fig. 4A). After treatment with ROSC for 12 h the phosphorylation of CDK7 was markedly reduced and almost

completely abolished after treatment with a lower dose (20 μ M) for a further 6 h (Fig. 4A). The loss of the site-specific phosphorylation of CDK7 was associated with its inactivation. The CDK7-mediated phos-

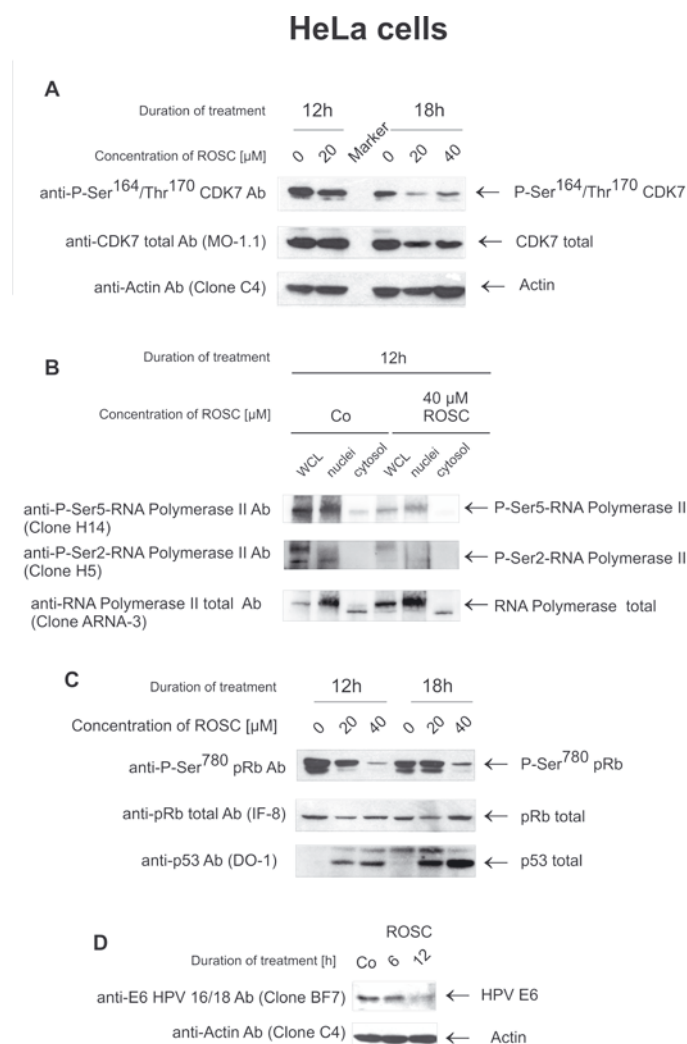


Figure 4. Repression of virally encoded E6 oncoprotein in asynchronously growing HeLa cells upon ROSC treatment.

Untreated and ROSC-treated HeLa cells were harvested and lysed in RIPA extraction buffer yielding whole cell lysates (WCLs). In some experiments cells were fractionated yielding nuclei and post-nuclear supernatant representing crude cytosolic fraction. Protein samples (WCLs and subcellular fractions) were loaded on 10% (B, C), 12% (A) or 15% (D) SDS/polyacrylamide slab gels. After protein separation and electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. Proteins and their phosphorylation status were examined by incubation with specific antibodies. Membranes were incubated with specific primary antibodies at appropriate final dilution and immune complexes were detected using appropriate HRP-conjugated secondary antibodies and enhanced chemiluminescent detection reagent ECL+ (Amersham International, Little Chalfont, Buckinghamshire, England). Equal protein loading was checked by immunoblotting with anti-actin antibodies. Chemiluminescence was analysed and documented using ChemiSmart 5100 equipped with a high resolution camera and image master software.

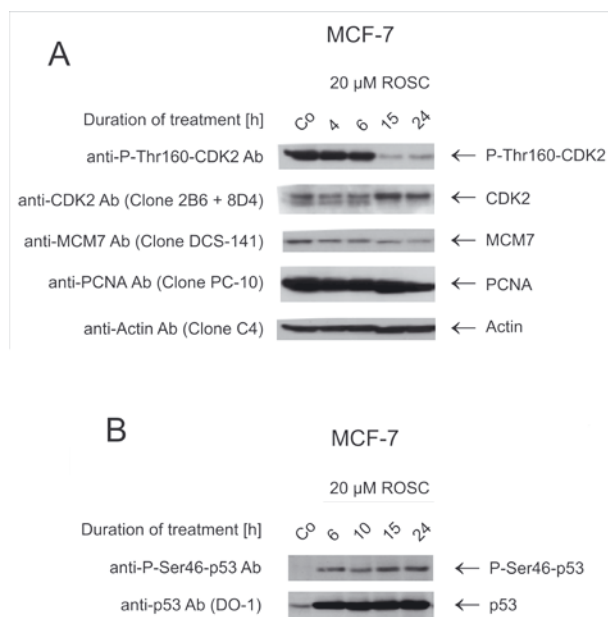


Figure 5. ROSC induces rapid phosphorylation of p53 protein at Ser46 in MCF-7 cells.

Exponentially growing MCF-7 cells were treated with 20 μ M ROSC for indicated periods of time. WCLs prepared from untreated controls and ROSC-treated cells were analysed by immunoblotting using antibodies recognizing P-Thr160 CDK2 and sequentially antibodies against total CDK2. The blot was sequentially incubated with monoclonal anti-MCM-7 and anti-PCNA antibodies (A). Second membrane was probed with antibodies reacting exclusively with p53 protein phosphorylated at Ser46 and sequentially with monoclonal DO-1 antibody recognizing total p53 protein (B). Immune complexes were detected as described in Fig. 4.

phorylation of RNA polymerase II was markedly reduced after exposure of HeLa cells to ROSC for 12 h (Fig. 4B). ROSC also inactivated CDK2 (not shown) resulting in the concentration-dependent diminution or even loss of phosphorylation of pRb protein (Fig. 4C). Surprisingly, ROSC increased the cellular level of p53 protein that was undetectable in untreated controls. The up-regulation of p53 was time- and concentration-dependent (Fig. 4C). Considering the fact that in HeLa cells wt p53 protein is antagonized by a virally-encoded E6 protein, its re-activation upon ROSC treatment would indicate that p53 escapes from the destructive action of its negative regulator or that the latter is repressed. The immunoblotting analysis confirmed that ROSC represses the expression of virally encoded proteins (Fig. 4D). The cellular level of E6 obviously decreased after 12 h of ROSC action (Fig. 4D) and after further 12 h E6 became undetectable (not shown). Exposure of HeLa cells to ROSC also induced apoptosis as evidenced by accumulation of hypoploid cells (Fig. 3) (see also Węsierska-Gadek *et al.*, 2008a; 2008b). The apoptosis rate was concentration-dependent. The inhibition of

cellular CDKs and RNA Pol II strongly affected the cellular levels of distinct pro-survival factors such as survivin, Bad, and Mcl-1 (not shown). Moreover, the ROSC-mediated reactivation of p53 protein also promoted initiation and execution of apoptosis.

Activation of p53 protein in ROSC-treated MCF-7 cells by phosphorylation at Ser46

Exposure of human MCF-7 cells to ROSC resulted in an inactivation of CDK2 as evidenced by loss of its phosphorylation at Thr160 (Fig. 5A). Interestingly, ROSC induced phosphorylation of p53 protein at Ser46 (Fig. 5B). The modification coincided with a strong up-regulation of the tumor suppressor protein thereby indicating that the post-translational modification considerably contributed to its stabilization. Determination of p53 protein stability in control and ROSC-treated MCF-7 cells confirmed this assumption. The p53 half-life increased approximately 15-fold after ROSC treatment (Węsierska-Gadek *et al.*, 2008a; 2008b) (not shown). ROSC did not affect PCNA level and only slightly diminished cellular concentration of MCM-7 protein after longer treatment (Fig. 5A). The latter observation coincides with a marked decrease of the proportion of S-phase cells (Fig. 3).

DISCUSSION

ROSC, a pharmacological CDK inhibitor, affects not only cell cycle kinases like CDK2 and CDK1 but additionally inhibits CDK7 (Havlicek *et al.*, 1997). CDK7, an intriguing enzyme complexed with cyclin H, cannot be simply classified (Fisher, 2005). It provides a direct link between regulation of the cell cycle and transcription, because it is both a CDK-activating kinase (CAK) and a constituent of the basal transcription factor TFIIF which phosphorylates serine residues within the heptapeptide repeat of the carboxy terminal domain (CTD) of RNA polymerase II (Palancade & Bensaude, 2003). The direct connection between the regulation of the activity status of CDKs and the transcription machinery maintained by CDK7 raises a number of questions. It is not clear how CDK7 is able to discriminate between two distinct substrates. CAK itself is in mammalian cells a trimeric complex consisting of the catalytic component p40^{MO15} designated as CDK7, a regulatory subunit cyclin H, and a RING finger assembly factor called ménage a trois, MAT1 (Fisher, 2005).

It has been shown that CDK7 forms a stable complex with cyclin H and MAT1 *in vivo* only when phosphorylated on either one of two residues (Ser164 or Thr170) in its T-loop. The phosphorylation status of the T-loop seems to modulate the sub-

strate specificity of CDK7 to favour CTD over CDKs. We monitored the impact of ROSC on the site-specific phosphorylation of CDK7 in HeLa cells. After treatment for 12 h the phosphorylation of CDK7 at critical amino acids decreased and after further 6 h it was abolished. The unphosphorylated CDK7 became unable to modify serine residues within the CTD. This coincided with a stepwise increase of p53 protein concentration and reduction of E6 level. These results indicate that ROSC efficiently represses the transcription of HPV-encoded proteins. These results also confirm previous observation that CDK inhibitory nucleoside analogs repress transcription from viral genomes (Schang *et al.*, 2005). Apart of the interference with the transcriptional machinery, ROSC also inhibited CDK2 (Wesierska-Gadek *et al.*, 2008b). The repression of E6 protein contributed to re-activation of p53 tumor suppressor protein. Considering the strong involvement of p53 protein in the induction of apoptosis and of cell cycle arrest, one might assume that reactivated p53 protein additionally enhanced the therapeutic effect of ROSC. Unlike HeLa cells, MCF-7 cells are more resistant to pro-apoptotic stimuli due to inactivation of caspase-3. ROSC arrested very rapidly MCF-7 cells at the G₂/M transition. These results are in concordance with our previous data (Wesierska-Gadek *et al.*, 2005; Wojciechowski *et al.*, 2003). Interestingly, ROSC strongly activated wt p53 *via* phosphorylation of Ser46. The p53 protein modified at Ser46 is a potent transcriptional activator of the *p53AIP1* gene (Oda *et al.*, 2000). The up-regulation of the p53AIP1 protein, a pro-apoptotic mitochondrial factor, resulted in induction of apoptosis in MCF-7 cells (Wesierska-Gadek *et al.*, 2005). We conclude that the dual action of ROSC enhanced its therapeutic potential on MCF-7 cells.

Our results evidence that CDK inhibitors targeting simultaneously CDKs involved in the regulation of cell cycle and transcription, such as ROSC, display high therapeutic potential. Their bi-functionality allows them to affect several pathways in malignant cells and even to overcome resistance to apoptosis.

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