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

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 (Beson 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 inhibitor 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 G_{S}/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 cycle progression of normal human MRC-5 and F2000 fibroblasts (Wesierska-Gadek 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 HeLaS\textsubscript{3} 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 (Havlícek 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 Corporation, 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 HeLaS\textsubscript{3} cervical carcinoma cells and their sensitivity to increasing concentrations of ROSC was determined by the CellTiter-GloTM 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 ± 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 appropriately 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

![Effect of ROSC on the number of viable MCF-7 cells](image1.png)

**Effect of ROSC on the number of viable MCF-7 cells**

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 µM 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 µM 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 inactivate 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 manner the expression of E6 and E7 oncoproteins.
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 µM) 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-
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 was undetectable in untreated controls. The up-regulation of p53 was time- and concentration-dependent (Fig. 4C). 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 Wesierska-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 (Wesierska-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 TFIIIF 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 p40MO15 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 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 inhibitors interfere 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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