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# Functional p53 in Cells Contributes to the Anticancer Effect of the Cyclin-Dependent Kinase Inhibitor Roscovitine

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## ABSTRACT

Inhibitors of cyclin-dependent kinases (CDKs) undergoing clinical trials as anticancer agents usually target several CDKs in cells. Some of them are also able to increase cellular levels of p53 protein and to activate p53-regulated transcription. To define the role of p53 in the anticancer effect of selective CDK inhibitors, two related compounds roscovitine and olomoucine II were studied. Roscovitine differs functionally from its congener olomoucine II only in the selectivity towards transcriptional CDK9. Action of both compounds on proliferation, cell-cycle progression, and apoptosis was examined in RPMI-8226 cells expressing the temperature-sensitive mutant of p53 and in MCF-7 cells with wild-type p53. Both compounds blocked proliferation, decreased phosphorylation of RNA polymerase II, downregulated antiapoptotic protein Mcl-1 in both cell lines in a dose-dependent manner, and also activated p53 in MCF-7 cells. Moreover, we showed that the anticancer efficiency of CDK inhibitors was enhanced by active p53 in RPMI-8226 cells kept at permissive temperature, where downregulation of Mcl-1, fragmentation of PARP-1, and increased caspase-3 activity was detected with lower doses of the compounds. The results confirm that functional p53 protein may enhance the anticancer activity of roscovitine that could be beneficial for anticancer therapy. J. Cell. Biochem. 107: 428–437, 2009. 

**KEY WORDS:** APOPTOSIS; CYCLIN-DEPENDENT KINASE; OLOMOUCINE II; p53; ROSCOVITINE

Several small molecular inhibitors of cyclin-dependent kinases (CDK) are currently undergoing clinical evaluation as a new generation of anticancer agents [Malumbres and Barbacid, 2001; Fischer and Gianella-Borradori, 2005; Malumbres et al., 2008]. Activities of CDKs are usually elevated in cancers, due to both genetic and epigenetic alterations of CDKs themselves or of the proteins they interact with, including activating cyclins and phosphatases (cdc25), inhibitors (INK4, CIP/KIP), and substrates (pRBs and E2Fs) [Malumbres and Barbacid, 2001]. These alterations provide a basis for pharmacological interventions by synthetic compounds specifically targeting hyperactive cell-cycle regulators in cancer cells.

The 2,6,9-trisubstituted purine derivative roscovitine belongs to the most advanced anti-CDK drugs [Meijer and Raymond, 2003; Fischer and Gianella-Borradori, 2005]. It has been long assumed that roscovitine, like many other CDK inhibitors, arrests and kills transformed cells as a direct consequence of the inactivation of cell-cycle CDKs. However, several recent articles also point to an involvement of the transcriptional CDK7 and CDK9 [Demidenko and Blagosklonny, 2004; MacCallum et al., 2005; Cai et al., 2006a; Gao et al., 2006; Hajduch et al., 2007]. Besides direct inhibition of CDK2 and CDK1 by roscovitine associated with cell-cycle arrest in $G_1$/S or $G_2$/M transitions, there are indirect mechanisms by which roscovitine can block the proliferation. For example, interaction

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with constitutively active CDK7, which phosphorylates the threonine residue in the activation loop of CDKs (e.g., Thr161 in CDK1), reduces levels of active CDKs [Hajduch et al., 2007]. An unphosphorylated activation loop closes the catalytic site and the activities of CDK1 and CDK2 then decrease. But most of the indirect mechanisms stem from changed gene expression profiles following treatment with roscovitine. Like several other CDK inhibitors, roscovitine also reduces levels of cyclins D1, B, and A, while simultaneously upregulating expression of the natural CDK inhibitor $p21^{waf1}$ [Kotala et al., 2001; Lu et al., 2001; Whittaker et al., 2004; Lacrima et al., 2005]. More recently, cDNA microarray analysis demonstrated that other important cell-cycle regulatory genes also change expression. For example, markedly reduced mRNA expression was found for mitotic regulators, including Aurora A and B, Polo-like kinase, Wee1 and Cdc25C [Whittaker et al., 2007]. These alterations also contribute to cell-cycle arrest and accumulating evidence suggests that roscovitine-sensitive CDK7 and CDK9 are responsible for the reduced expression [Whittaker et al., 2004; Lacrima et al., 2005; MacCallum et al., 2005].

CDKs 7 and 9 stimulate initiation and elongation of mRNA transcription, respectively, by phosphorylating the C-terminal domain of RNA polymerase II. Suppressed transcription and decreased phosphorylation of RNA polymerase II were documented in cells treated not only with roscovitine, but also with other CDK inhibitors [Ljungman and Paulsen, 2001; Demidenko and Blagosklonny, 2004; Whittaker et al., 2004]. The transcripts that are most sensitive to CDK9 inhibition are those with short half-lives such as cell-cycle regulators, mitotic kinases, NF-κB-regulated genes, and apoptosis regulators, because their levels decrease rapidly when initiation and elongation of transcription are inhibited. One of them is MDM2, an E3-ubiquitin ligase negatively regulating tumor suppressor p53 protein level [Momand et al., 1992]. p53 is a stress-inducible transcription factor that controls hundreds of genes involved in a variety of cellular functions, including cell-cycle arrest, DNA repair, and apoptosis [Wei et al., 2006]. Due to amplification or overexpression, MDM2 contributes to cancer development. Disruption of the interaction between p53 and MDM2 provides a rationale for therapeutic p53 activation in cancers bearing wild-type p53 gene. Interestingly, an alternative mechanism of anticancer activity of roscovitine and some other CDK inhibitors is based on accumulation of tumor suppressor protein p53 in nuclei in a transcriptionally active form [David-Pfeuty, 1999; Kotala et al., 2001; Lu et al., 2001; Wojciechowski et al., 2003; Demidenko and Blagosklonny, 2004; Wesierska-Gadek et al., 2005]. The effect may be due to downregulation of MDM2, as reduced expression of MDM2 helps to stabilize p53 level. An increase of p53-dependent transcription is also frequently observed, with the concomitant accumulation of $p21^{waf1}$, one of the endogenous CDK inhibitors [Kotala et al., 2001; Lu et al., 2001].

In this study, we describe effects of roscovitine in human cancer cell lines differing in p53 status. As shown recently, it has a greater potency against p53 wild-type cell lines than against p53 mutant cell lines [McClue et al., 2002; Krystof et al., 2005]. To define the role of p53 in the anticancer effect of CDK inhibitors, we selected the RPMI-8226 cell line, expressing a temperature-sensitive mutant of p53 that allowed us to study the function of p53 in the same genetic background under different temperatures, and the MCF-7 cell line with wild-type p53. Structurally distinct compounds with surmised functional similarities (i.e., with a common molecular target) are often used for understanding and validating their molecular impact. However, although these distinct compounds usually help to reveal off-target effects, they may not only differ substantially in affinity to the primary target, but also in other features such as polarity, stability, and cellular uptake that could unknowingly limit the comparative work. Therefore, our approach has been based on use of two structurally related compounds, the functional analogues roscovitine and olomoucine II, that strongly and equally inhibit kinases CDK2 and CDK7. Importantly for this study, both congeners differ in selectivity towards transcriptional CDK9, which is more sensitive to olomoucine II [Krystof et al., 2005]. Based on our data, we demonstrate a crucial role of CDK9 in the upregulation of p53 and p53-connected anticancer properties of roscovitine. Our results provide another insight into the cellular effects of roscovitine and support its therapeutic application in cancers with wild-type p53.

## MATERIALS AND METHODS

### DRUGS AND ANTIBODIES

*R*-roscovitine (6-benzylamino-2-[(*R*)-(1-ethyl-2-hydroxyethylamino)]-9-isopropylpurine) and *R*-olomoucine II (6-(2-hydroxybenzylamino)-2-{[(1*R*)-(hydroxymethyl)propyl]amino}-9-isopropylpurine) were synthesized according to published procedures [Havlicek et al., 1997; Krystof et al., 2002]. For cell treatment, compounds were made up as 100 mM stocks in dimethylsulfoxide (DMSO) and diluted prior to application in culture media. The maximum concentration of DMSO in the medium never exceeded 0.1%.

The following specific antibodies were used to detect the relevant proteins: anti-p53 (clone DO-1), anti-cyclin D1, anti-CDK4, anti-MDM2 (clone 2A9), anti-PCNA (clone PC-10), and anti-$p21^{waf1}$ (clone 118, all gifts from B. Vojtesek); anti-α-tubulin (clone DM1A; Sigma–Aldrich, Prague, Czech Republic); anti-Mcl-1 (clone S-19), anti-PARP-1 (clone F-2), anti-cyclin E (clone HE12), and anti-bcl-2 (clone 100; all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-RNA polymerase II (clone ARNA-3; Millipore, Prague, Czech Republic); anti-RNA polymerase II phosphorylated on Ser-5 (clone H14) and anti-RNA polymerase II phosphorylated on Ser-2 (clone H5, both from Abcam, Cambridge, UK); fluorescein-labeled anti-BrdU (Becton–Dickinson, Prague, Czech Republic).

### CELL CULTURES AND VIABILITY ASSAY

Human MCF-7 breast carcinoma and RPMI-8226 multiple myeloma cell lines purchased from American Type Culture Collection were maintained in a humidified $CO_2$ incubator at 37 °C in DMEM or RPMI medium, respectively, supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Viability assays were performed in triplicates in 96-well microtiter plates with cells seeded at densities appropriate for their respective cell sizes and doubling times. Twelve hours after seeding, tested compounds in threefold dilutions were added in triplicate and the treatment lasted continuously for 72 h. At the end of this period, the cells were fed for 1 h with Calcein AM solution

(Invitrogen, Carlsbad, CA) and the fluorescence of the live cells was measured at 485/538 nm (ex/em) with a Fluoroskan Ascent microplate reader (Labsystems, Helsinki, Finland). The drug concentrations lethal to 50% of the cells ($IC_{50}$ values) were determined from the dose–response curves using GraphPad Prism software (GraphPad Software, La Jolla, CA).

### BrdU INCORPORATION AND CELL-CYCLE ANALYSIS

Before harvesting, cells were pulse labeled with 10 μM 5-bromo-2′ deoxyuridine (BrdU) for 30 min. The cells were trypsinized, fixed with ice-cold 70% ethanol, incubated on ice for 30 min, washed with PBS, and resuspended in 2 M HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$, the cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. They were then stained with fluorescein-labeled anti-BrdU antibody for 30 min at room temperature in the dark. The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/ml) and RNAse A (0.5 mg/ml) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur; Becton–Dickinson).

### CASPASES-3/7 ASSAY

Treated cells were harvested by centrifugations and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. The homogenates were clarified by centrifugation at 10,000*g* for 20 min at 4°C, the proteins were quantified by the Bradford method and diluted to the same concentration. Lysates were then incubated for 1 h with 100 μM Ac-DEVD-AMC as substrate (Sigma–Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 μM Ac-DEVD-CHO as a caspase-3/7 inhibitor (Sigma–Aldrich). The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 346/442 nm (ex/em).

### SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

For immunoblotting, harvested cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 μM PMSF, 1 mM DTT, 10 μg/ml of aprotinin and leupeptin). Proteins in lysates were quantified by the Bradford method and then diluted with Laemmli electrophoresis buffer. Proteins were then separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Prague, Czech Republic) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% low-fat milk and 0.1% Tween-20 in PBS or in 3% BSA and 0.1% Tween-20 in TBS (for the detection of phosphoproteins), respectively, for 2 h and probed with the specific primary antibodies overnight. After washing in PBS/TBS and PBS/TBS with 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and vizualized with chemiluminescent detection reagent ECL+ (Amersham Biosciences, Prague, Czech Republic). To confirm equal protein loading, immunodetection was performed with the anti-α-tubulin monoclonal antibody.

## RESULTS

### ANTIPROLIFERATE EFFECTS OF ROSCOVITINE AND OLOMOUCINE II

The MCF-7 breast cancer and multiple myeloma RPMI-8226 cell lines were treated with the CDK inhibitors roscovitine and olomoucine II, and the percentage of surviving cells was measured by the Calcein AM viability assay. Cells were incubated with increasing concentrations of both drugs for 72 h and then the $IC_{50}$ values were determined. As shown in Figure 1, neither roscovitine nor olomoucine II affected viability of cells at low concentrations

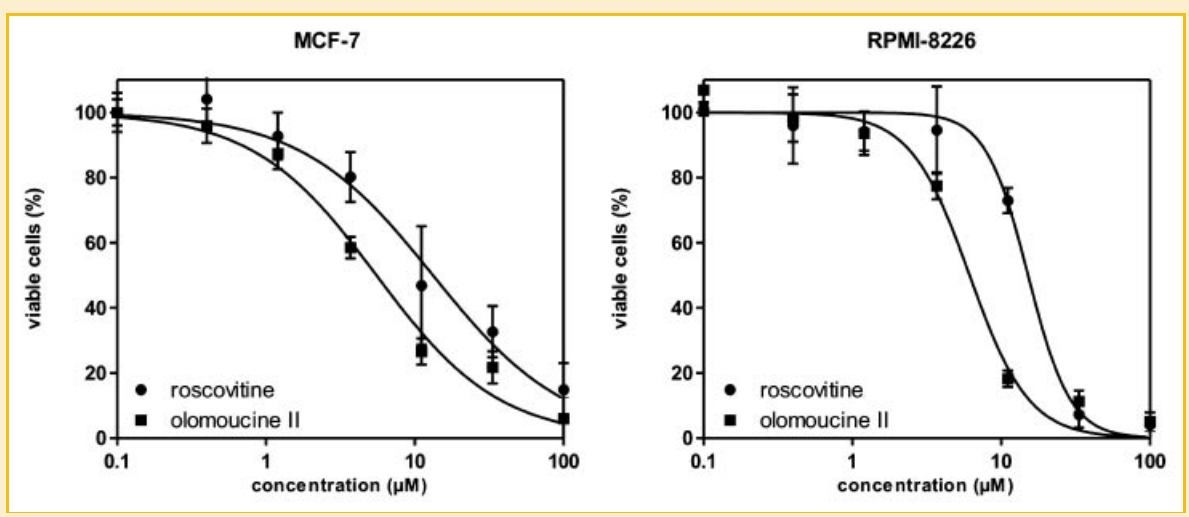


Fig. 1. Roscovitine and olomoucine II reduce the number of living cells. Human MCF-7 breast cancer and RPMI-8226 multiple myeloma cell lines were treated for 72 h with increasing concentrations of roscovitine and olomoucine II. Then, the number of viable cells was determined by a Calcein AM assay. Results represent the average ± SD for three independent experiments.

over the 72 h incubation period. The $IC_{50}$ values are 15.2 μM for roscovitine and 6.1 μM for olomoucine II in RPMI-8226 cells. The sensitivity of the MCF-7 cell line is comparable to that of multiple myeloma; the $IC_{50}$ of roscovitine is 13.3 and 5.5 μM for olomoucine II in MCF-7 cells. The results show that olomoucine II is at least twofold more effective than roscovitine in both cell lines.

### CDK INHIBITORS ARREST CELL CYCLE IN MULTIPLE PHASES

To further test the antiproliferative effects of CDK inhibitors, we treated both cell lines with either roscovitine or olomoucine II for 24 h and analyzed their dose-dependent effect on the cell division cycle. The cells, pulse labeled with BrdU, were doubly stained with PI and anti-BrdU antibody and analyzed by flow cytometry. As shown in Figure 2, CDK inhibitors arrested both cell lines in $G_2$/M phases and decreased the size of their S-phase populations. In addition, RPMI-8226 cells also partially accumulated in the $G_1$ phase upon treatment with higher concentrations of both inhibitors. Importantly, both CDK inhibitors also markedly decreased the population of cells actively replicating DNA (i.e., BrdU-positive cells).

### ROSCOVITINE AND OLOMOUCINE II INDUCE CASPASE-DEPENDENT CELL DEATH

The analysis of the DNA profiles of RPMI-8226 myeloma cells revealed an accumulation of sub-$G_1$ cells treated with both CDK inhibitors indicating that cells undergo apoptosis. The appearance of hypoploid cells is usually attributable to caspase-dependent fragmentation of chromatin. Therefore, we determined the activity of caspase-3/7 in RPMI-8226 cells exposed to roscovitine or olomoucine II using a fluorogenic substrate Ac-DEVD-AMC. In the first series of experiments, cells were treated in a time-dependent manner with fixed drug concentrations exceeding their $IC_{50}$ values (40 μM roscovitine and 20 μM olomoucine II). Roscovitine strongly induced the activity of caspase-3/7; after treatment for only 3 h a threefold increase of the effector caspases was observed compared with the untreated control; this increased to a sevenfold increase after 6 h (Fig. 3A). Unlike roscovitine, olomoucine II only weakly affected the activity of caspases-3/7; a threefold enhancement of the activity was detected after 6 h and after longer treatment the caspase-3/7 activity decreased.

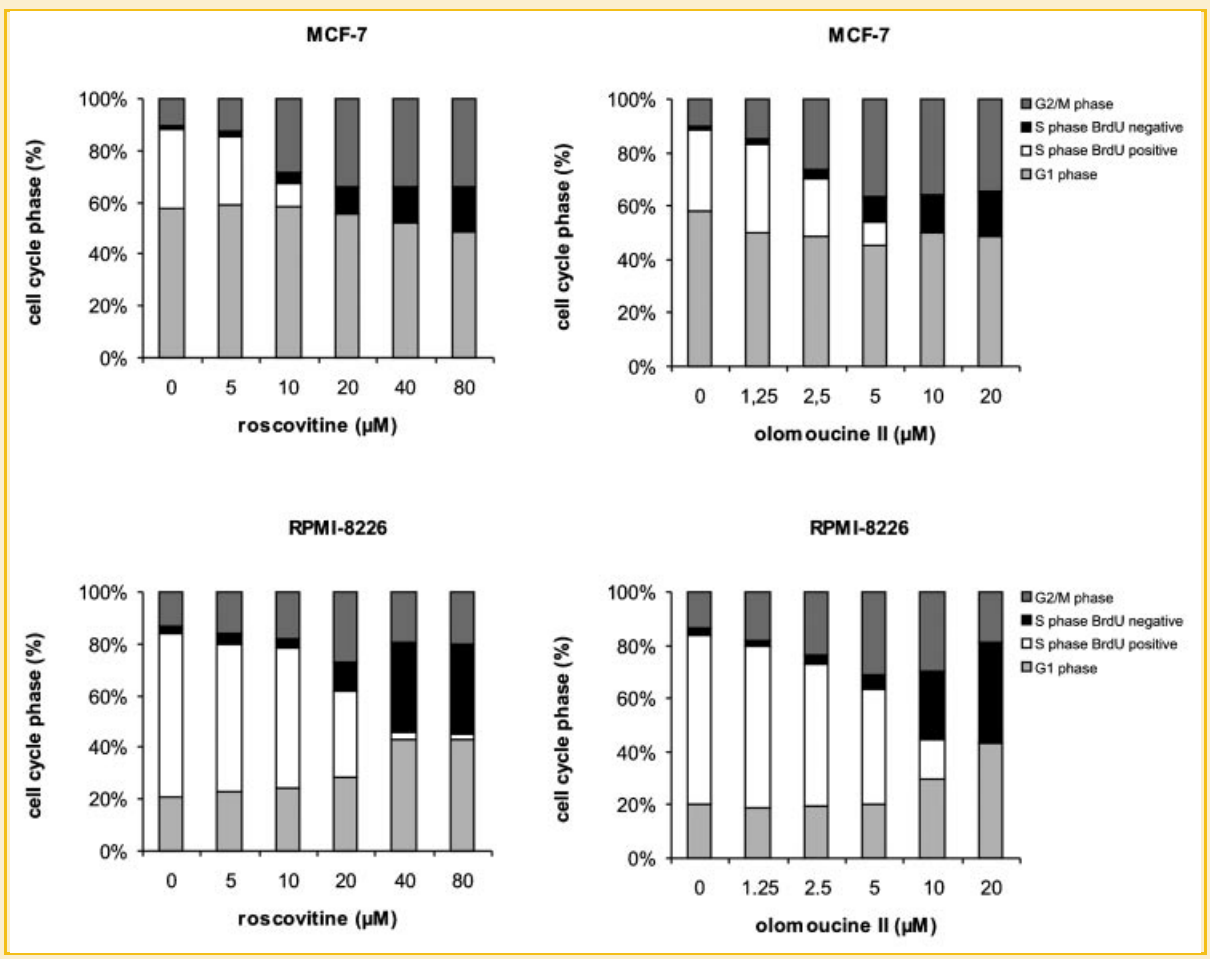


Fig. 2. Roscovitine and olomoucine II induce cell-cycle arrest. MCF-7 and RPMI-8226 cells were treated for 24 h with increasing concentrations of roscovitine and olomoucine II. Half an hour before the end point, BrdU was added to the culture media. The cells were collected, fixed, and stained with propidium iodide and anti-BrdU antibodies. DNA content and the percentage of BrdU-labeled cells were quantified by flow cytometry.

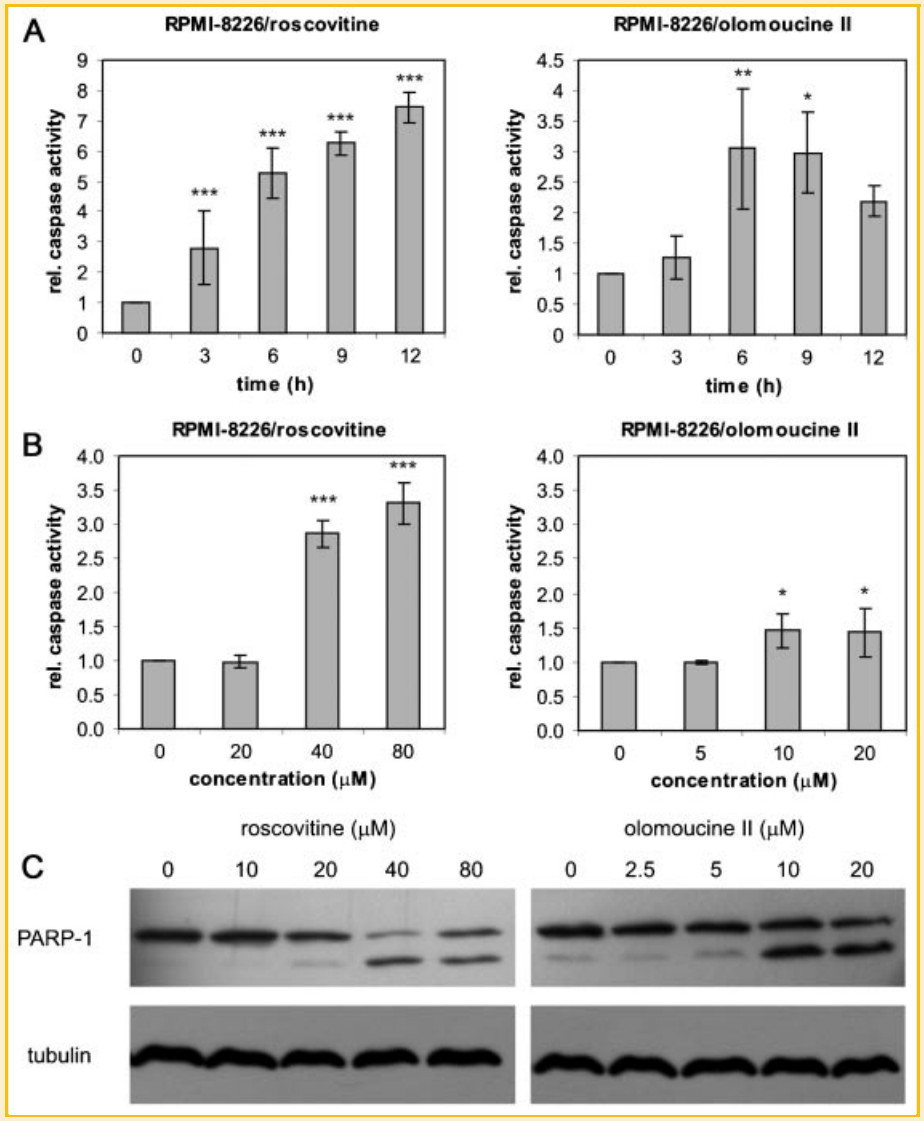


Fig. 3. Roscovitine and olomoucine II cause cell death. RPMI-8226 cell line was treated in a time (A) or concentration-dependent (B,C) manner with roscovitine and olomoucine II, harvested and whole cell lysates were prepared. The activities of caspases-3/7 were measured using specific fluorogenic peptide substrate Ac-DEVD-AMC (A,B), or the fragmentation of PARP-1 was detected by immunoblotting (C). The significance (treatment vs. control) was determined using Dunnett's multiple comparison test: $^{*}P < 0.05$ (significant); $^{**}P < 0.01$ (very significant); $^{***}P < 0.001$ (extremely significant).

In a concentration-dependent experiment, the cells were treated for 24 h. As shown in Figure 3B, 40 and 80 μM roscovitine induced a strong activation of procaspases-3/7, whereas olomoucine II activated procaspases-3/7 only weakly in RPMI-8226 cells.

Monitoring of the cleavage of PARP-1, a nuclear target of caspase-3, confirmed the above results. An appearance of the caspase-3-cleaved PARP-1 fragment at 89 kDa after cell exposure to 40 μM roscovitine was associated with a diminution of its full-length form (Fig. 3C; left panel). Fragmentation of PARP-1 was also detected after treatment of RPMI-8226 cells with olomoucine II at higher doses (10 and 20 μM). However, the intensity of full-length PARP-1 remained almost unchanged (Fig. 3C; right panel).

Taken together, our results clearly evidence that roscovitine strongly activates pro-caspases-3/7 in RPMI-8226 cells in a time- and concentration-dependent manner. However, the results for olomoucine II are inconsistent and its stimulatory effect on pro-caspase-3/7 is only weak. Our data suggest that olomoucine II may initiate cell death by a mechanism different from that of roscovitine.

## EXPRESSION OF CELL-CYCLE AND APOPTOTIC PROTEINS

To better understand the mechanisms leading to the cell-cycle arrest and induction of apoptosis by roscovitine and olomoucine II, we next analyzed changes in selected proteins by immunoblotting (Fig. 4). In MCF-7 cells, treatment with equiactive doses of roscovitine and olomoucine II (20 and 5 μM, respectively) resulted in a significant reduction in CDK4 expression and when higher doses were used, the level of its positive regulator cyclin D1 also declined. In RPMI-8226 cells, the level of CDK4 was rapidly decreased, but cyclin D1 expression remained unchanged. Reduction of cyclin E, a regulatory subunit of CDK2 controlling $G_1$/S phase transition, also

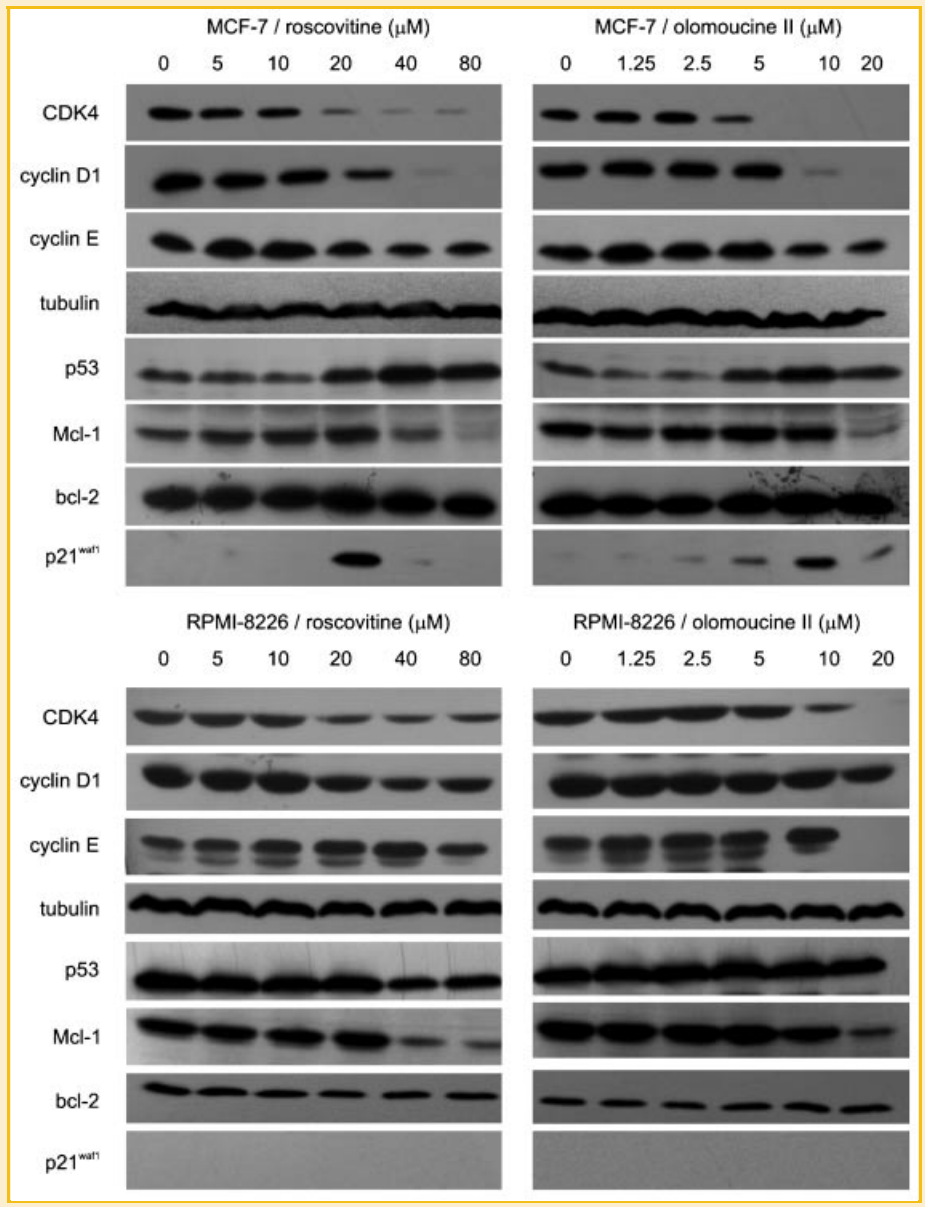


Fig. 4. Roscovitine and olomoucine II change the expression of the cell-cycle proteins and regulators of apoptosis. Cell lines were treated in a concentration-dependent manner with roscovitine and olomoucine II for 24 h, harvested, and whole cell lysates were prepared. The changes in the expression of various cell-cycle regulators in MCF-7 cell line expressing wild-type p53 and RPMI-8226 cell line with mutant p53 were analyzed by immunoblotting.

occurred in both cell lines. These observations correlate with flow cytometry results, where the rapid $G_1$ block and declined S phase population (in RPMI-8226 cells) and increasing of $G_2$/M arrest and decreased S phase (in MCF-7) were seen following treatment with CDK inhibitors.

In line with previously published data, both roscovitine and olomoucine II were also able to cause the accumulation of p53 in MCF-7 cells and activate $p21^{waf1}$ expression (Fig. 4). Interestingly, $p21^{waf1}$ expression diminished with the highest concentrations of CDK inhibitors, probably as a result of inhibition of general mRNA transcription. Interestingly, in RPMI-8226 cells with inactive p53, levels of neither p53 nor $p21^{waf1}$ changed. In contrast, the antiapoptotic protein Mcl-1 decreased in both cell lines following treatment, whereas no changes were detected in the levels of another antiapoptotic factor bcl-2 (Fig. 4) or proapoptotic protein bax (not shown).

## DEPHOSPHORYLATION OF THE RNA POLYMERASE II

Previous studies have shown that treatment of cells with roscovitine leads to dephosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II, particularly at serine 2 and 5, which consequently decreases transcription rate. The phosphorylation of the CTD of RNA polymerase II is caused by two CDKs, namely CDK9, which is specific for Ser-2 phosphorylation, and CDK7, which phosphorylates both sites. Also, the phosphorylation at Ser-5, but

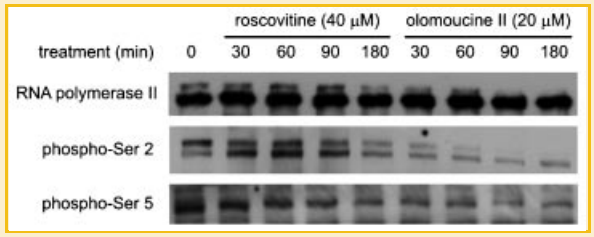


Fig. 5. Roscovitine and olomoucine II modulate expression of RNA polymerase II and its phosphorylation status in RPMI-8226 cells. Cells were incubated with drugs at the indicated time points, harvested, and whole cell lysates were analyzed by immunoblotting.

not at Ser-2, is responsible for prevention of RNA polymerase II ubiquitylation.

To confirm our assumption that not only roscovitine but also olomoucine II inhibits CTD phosphorylation, RPMI-8226 cells were treated with both CDK inhibitors in a time-dependent manner (Fig. 5). The expression of total RNA polymerase II did not change during treatment with both CDK inhibitors for 3 h. However, phosphorylated Ser-2 was markedly reduced after 60 min in olomoucine II-treated cells, while only moderate roscovitine-induced diminution of the Ser-2 phosphorylation was detected after 1.5 h and at its twofold higher concentration. The longer exposure (12 or 24 h) of cells to the drugs resulted in degradation of total RNA polymerase II level.

### REACTIVATION OF WILD-TYPE p53 PROTEIN IN RPMI-8226 CELLS SENSITIZES THEM TO INDUCTION OF APOPTOSIS

The cell lines examined in this study differ in their p53 status; MCF-7 cells express wild-type p53, while in RPMI-8226 cells bearing temperature-sensitive mutant of p53 (E285K), p53 protein switches between mutant and wild-type conformation in a temperature-dependent manner. At restrictive (37 °C) temperature mutated p53 protein occurs. After shift to a permissive (30 °C) temperature, the protein is folded to functional p53. To analyze the functional involvement of p53 in the efficiency of the treatment with CDK inhibitors, we cultivated RPMI-8226 cells independently at two different temperatures (restrictive or permissive), and exposed them to increasing doses of roscovitine or olomoucine II. The cell lysates were prepared and the levels of cell-cycle regulators were analyzed by immunoblotting.

Both CDK inhibitors caused accumulation of wild-type p53 protein in cells maintained at permissive temperature, but did not change the levels of mutant p53 in cells cultivated at restrictive temperature. This indicates that both CDK inhibitors promote stabilization of wild-type p53 protein. Moreover, the monitoring of the expression of p53 targets such as $p21^{waf1}$ and MDM2 revealed their appearance following treatment with CDK inhibitors solely in cells maintained at permissive temperature, but not in cells kept at restrictive temperature (Fig. 6).

Remarkably, cells under permissive conditions underwent apoptosis even at lower concentrations of roscovitine and olomoucine II (Fig. 6). We have observed fragmentation of PARP-1 to occur in cells treated with about twofold lower drug concentrations that in cells kept in restrictive temperature (Fig. 6A). Similarly, expression of antiapoptotic protein Mcl-1 was also suppressed more efficiently at 30 °C by both inhibitors (Fig. 6A). More importantly, activities of caspases-3/7 extracted from cells kept under different conditions significantly differed, that is, they were higher in lysates prepared from cells kept at 30 °C (Fig. 6B). These data evidence that wild-type p53 protein expressed in RPMI-8226 cells cultured at permissive temperature facilitates induction of apoptosis upon treatment with roscovitine and olomoucine II.

## DISCUSSION

Observations reported in many articles have provided the rationale for therapeutical applications of roscovitine, a 2,6,9-trisubstituted purine inhibitor of cyclin-dependent kinases CDK1, CDK2, CDK3, CDK7, and CDK9. This drug has entered clinical trials as a potential agent for treatment of several different cancers, including B-cell malignancies, lung and breast cancers [Meijer and Raymond, 2003; Meijer et al., 2006]. Roscovitine has been shown to arrest the cell cycle in proliferating cells, a response associated with pRb dephosphorylation [Whittaker et al., 2004]. The antiproliferative effects of roscovitine are caused by its ability to target multiple CDKs simultaneously rather than only one of them. The ability to induce cell arrest and death through depletion of the activity of a single CDK is weak and often cell line dependent, but cooperative pharmacological inactivation of several CDKs results in stronger antiproliferative effects in cancer cell lines [Cai et al., 2006b]. Similarly, recent genetic experiments demonstrated that multiple CDKs are not essential for mouse embryonic cells, where only mitotic CDK1 is required [Santamaría et al., 2007]. Interestingly, in cells lacking multiple CDKs, unusual compensatory complexes of CDK1 with cyclins E or D were detected [Aleem et al., 2005; Cai et al., 2006b; Santamaría et al., 2007]. Therefore, CDK1 can apparently compensate for the lack of, but itself cannot be replaced by, any interphase CDK in normal cells [Santamaría et al., 2007]. The situation in human adult or transformed cells is still not completely clear, but available data suggest that only combined inhibition of CDK1, CDK2, and CDK9 in some cancer cell lines significantly decreases cell proliferation and enhances apoptosis, too. Notably, CDK1, CDK2, and CDK9 represent a rational subset of CDK family members for drug targeting [Cai et al., 2006a,b] and this subset overlaps well with the selectivity of roscovitine [McClue et al., 2002; Krystof et al., 2005].

In line with published results, we found that not only roscovitine but also olomoucine II arrested the cell cycle in MCF-7 and RPMI-8226. Both CDK inhibitors blocked the $G_2$/M transition and brought about a concentration-dependent reduction in BrdU incorporation. Furthermore, the blocking of $G_1$ was evident at higher concentrations of both drugs in the RPMI-8226 cell line. The cell-cycle arrest was accompanied by decreased levels of cyclin D1, cyclin E, and CDK4 in both cell lines, as well as enhanced expression of transcription factor p53 and its downstream target $p21^{waf1}$ in MCF-7. There were no substantial differences in the effects of both compounds, other than the higher efficiency of olomoucine II over roscovitine attributable to stronger CDK9 inhibition.

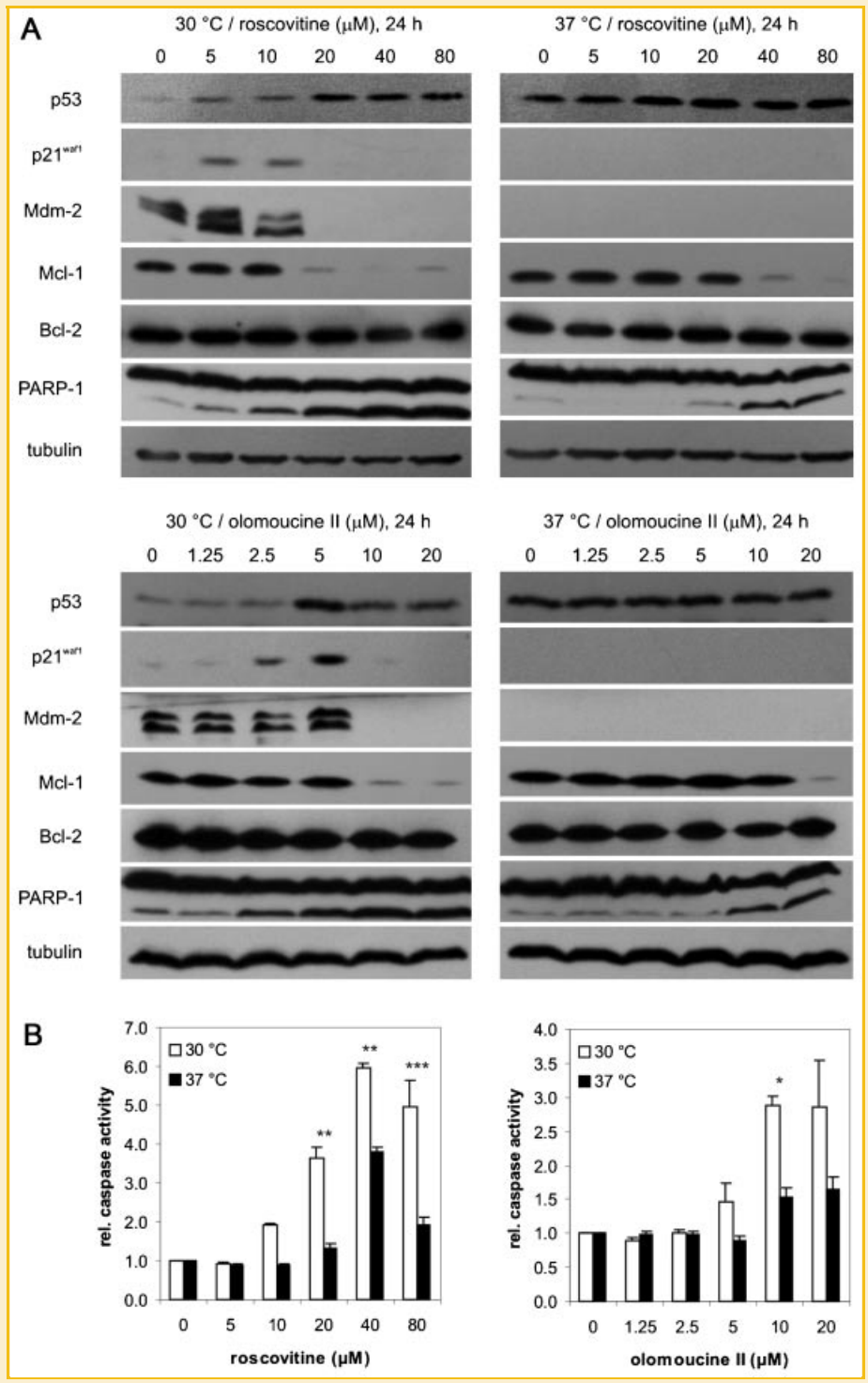


Fig. 6. Roscovitine and olomoucine II induce reactivation of temperature-sensitive mutant p53 in RPMI 8826. RPMI-8226 cells were treated for 24 h in a concentration-dependent manner with roscovitine and olomoucine II at 30 or 37°C, harvested, and whole cell lysates were prepared. The expression of cell-cycle regulators and proteins involved in apoptosis were detected by immunoblotting (A). The activities of caspases-3/7 in treated cells were measured using specific fluorogenic peptide substrate Ac-DEVD-AMC (B). The significance (treatment at 30°C vs. 37°C) was determined using Bonferroni's multiple comparison test: $^{*}P < 0.05$ (significant); $^{**}P < 0.01$ (very significant); $^{***}P < 0.001$ (extremely significant).

However, the exact mechanism by which roscovitine induces apoptosis has not been clearly defined and it is still uncertain which CDKs (or other targets) are critical for its anticancer effects and to what extent. Several studies have shown that CDK9 is an important target of some pharmacological CDK inhibitors, including roscovitine, flavopiridol, SU9516, and AZ703 [Gojo et al., 2002; MacCallum et al., 2005; Raje et al., 2005; Gao et al., 2006; Cai et al., 2006a]. As well as others, all these drugs are capable of

inhibiting phosphorylation of the C-terminus of RNA polymerase II and therefore act as global transcriptional repressors. RNA polymerase II is regulated by phosphorylation of Ser-5 and Ser-2 within the CTD by CDK7 and CDK9 and probably also by cell-cycle-related CDK1 and CDK2 [Cai et al., 2006b]. Hence, the major impact of these pharmacological inhibitors is manifested primarily on short-lived proteins such as Mcl-1, XIAP, or $p21^{waf1}$. Of these, Mcl-1 plays a critical role in the survival of cancer cells being an important antiapoptotic protein [MacCallum et al., 2005; Yang-Yen, 2006]. Both Mcl-1 protein and its mRNA have very short half-lives and therefore repression of transcription leads to its relatively rapid elimination from cells and promotes apoptosis. Downregulation of Mcl-1 by roscovitine and olomoucine II in the two cell lines shown here is therefore consistent with observations by others [Gojo et al., 2002; MacCallum et al., 2005; Raje et al., 2005; Gao et al., 2006]. Indeed, both CDK inhibitors were able to induce apoptosis in RPMI-8226 cells, as evidenced by increased caspase-3/7 activity and fragmentation of PARP-1. Surprisingly, despite weaker caspase-3/7 activation by olomoucine II, the cells were still more sensitive to olomoucine II (cf. to Fig. 1) as shown by enhanced PARP-1 fragmentation. The results suggest that different cell death pathways (proteases) may be involved in the apoptosis induced by both structurally related CDK inhibitors. Due to different inhibitory potency of the compounds towards CDK9, we may speculate about the role of CDK9 (or an unknown target of the inhibitors) in caspase-3/7 activation and alternative mechanisms of cell death. The existence of an alternative mechanism of cell death would explain why MCF-7 cells also respond to olomoucine II more rapidly than to roscovitine, even though they lack functional caspase-3 [Simstein et al., 2003].

Inhibition of RNA polymerase II-dependent transcription also leads to accumulation of p53 by blocking its degradation by MDM2 [Kotala et al., 2001; Lu et al., 2001]. Recently, it was described that p53 restoration can be strongly potentiated by combinations of p53-activating agents with different actions, such as nutlin-3a, a specific inhibitor of MDM2, with nongenotoxic CDKs inhibitors, like DRB and roscovitine [Cheok et al., 2007]. We showed here the influence of roscovitine and its derivative olomoucine II on reactivation of the temperature-sensitive E285K mutant of p53 in RPMI-8226 cells. The lower temperature allows stabilization of active p53 and subsequent expression of $p21^{waf1}$, enhanced by roscovitine and olomoucine II, as also shown here. Simultaneously, reduced expression of antiapoptotic Mcl-1 and fragmentation of PARP-1 were observed with at least twofold lower doses of the compounds at permissive conditions. Nearly the same effect of both drugs was also observed following measurement of caspase-3/7 activity, together suggesting that cells die more easily if they possess functional p53, as suggested by our previous study [Krystof et al., 2005].

However, blockage of transcription and accumulation of p53 resulting from diminished levels of MDM2 is definitely not the only reason for induction of programmed cell death by CDK inhibitors [O'Hagan and Ljungman, 2004; Wesierska-Gadek et al., 2005, 2007]; specific posttranslational modifications of p53 are necessary for its particular functions. It has been evidenced that roscovitine activates HIP2 kinase, modifying p53 at Ser-46, and that overexpression of HIP2 kinase increases rate of apoptosis in MCF-7 cells, involving p53AIP1 protein, the downstream target of p53 [Wesierska-Gadek et al., 2007]. This pathway probably helps to overcome lack of functional caspase-3 in MCF-7 cells and increase the efficacy of drug-induced apoptosis. Similarly, our results also indicate that CDK inhibitors are able to induce different apoptotic pathways, depending on the status of p53 and/or caspase-3 in the two cell lines used here.

Moreover, it seems to be possible that interplays between p53 and other antiapoptotic/prosurvival molecules also contribute to roscovitine-induced apoptosis in cancer cells. For example, recent article suggests that p53-dependent apoptosis is potentiated by NF-κB suppression and shows that both these pathways are targeted simultaneously by roscovitine [Dey et al., 2008]. Or alternatively, it was shown recently that mitochondrial p53 displaces Mcl-1 from the complex with Bak upon cell stress, resulting in release of cytochrome *c* from mitochondria and induction of apoptosis [Leu et al., 2004]. p53 negatively regulates translationally controlled tumor protein TCTP, which normally binds to Mcl-1 and thus prevents its ubiquitinylation by ARF-BP/Mule [Zhong et al., 2005]. Stabilized and activated p53 may therefore influence Mcl-1 activity directly by disrupting its pro-survival function in mitochondria and indirectly by allowing its ubiquitinylation [Zhong et al., 2005]. Our finding confirms that simultaneous inactivation of the CDKs involved in the regulation of the cell cycle, as well as transcription, seems to be beneficial for anticancer therapy and further suggests that active p53 may enhance the anticancer activity of roscovitine through multiple mechanisms.

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