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The Cyclin-Dependent Kinase Inhibitor Roscovitine Inhibits RNA Synthesis and Triggers Nuclear Accumulation of p53 That Is Unmodified at Ser15 and Lys382

MATS LJUNGMAN and MICHELLE T. PAULSEN

Department of Radiation Oncology, Division of Cancer Biology, University of Michigan Comprehensive Cancer Center (M.L., M.T.P.), and Program in Cellular and Molecular Biology, University of Michigan Medical School (M.L.), Ann Arbor, Michigan,

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ABSTRACT

Roscovitine has been shown to induce the accumulation of the tumor suppressor p53, to arrest cells in the G_1 and G_2/M phases of the cell cycle, and to induce apoptosis in human cells. Although these cellular effects of roscovitine are thought to be caused directly by its specific inhibition of cyclin-dependent kinases, other mechanisms may contribute as well. In this study, we investigated whether roscovitine interferes with transcription in human cells. We have previously shown that blockage of transcription is a trigger for the induction of p53 and apoptosis in human fibroblasts. Here we show that mRNA synthesis is suppressed significantly by roscovitine in human cells. Furthermore, our results suggest that the mechanism by which roscovitine inhibits RNA synthesis involves the inhibition of the phosphorylation of the carboxyl-terminal domain of RNA polymerase II. Cells treated with roscovitine at doses that affected transcription were found to accumulate p53 in the nucleus; curiously, however, the nuclear accumulation of p53 was not accompanied by modifications at either the Ser15 or Lys382 sites of p53. We conclude that roscovitine is a potent inhibitor of RNA synthesis and that this inhibition may be responsible for the accumulation of nuclear p53.

2-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (roscovitine) is a potent but reversible inhibitor of Cdc2, Cdk2, Cdk5, and Cdk7 by acting as a competitor for ATP binding (Meijer et al., 1997; Hajduch et al., 1999; Sielecki et al., 2000). Roscovitine has been shown to arrest cells in the G_1 and G_2/M phases of the cell cycle (Meijer et al., 1997), inhibit DNA synthesis (Yakisich et al., 1999; Schang et al., 2000) cause nucleolar fragmentation (David-Pfeuty, 1999), and induce apoptosis in human cell lines (Mgbonyebi et al., 1999; Somerville and Cory, 2000). Interestingly, roscovitine-treated cells undergo apoptosis in all phases of the cell cycle (David-Pfeuty, 1999). Because of these cell growth-inhibiting activities, roscovitine is being considered as a potential anticancer agent (Yakisich et al., 1999; Buolamwini, 2000; Edamatsu et al., 2000).

It was recently shown that roscovitine induces p53 in human cells (David-Pfeuty, 1999). Because both Cdc2 and Cdk2 can phosphorylate the Ser315 site of p53 in vitro (Wang and Prives, 1995; Luciani et al., 2000; Blaydes et al., 2001), it is possible that the accumulation of p53 after inhibition of these kinases by roscovitine could be caused by loss of Ser315 phosphorylation (David-Pfeuty, 1999; Ljungman, 2000). In fact, it has been shown that phosphorylation of the Ser315 site of p53 by the Cdc2 and Cdk2 kinases attenuates tetramerization of p53 (Sakaguchi et al., 1997) and may make p53 more vulnerable to proteasome-mediated degradation (Lin and Desiderio, 1993). Tetramerization of p53 is thought to result in the shielding of the nuclear export signal of p53 and thereby stimulate nuclear accumulation of p53 (Stommel et al., 1999). By blocking phosphorylation of Ser315, roscovitine may favor the dephosphorylation of Ser315 by the Cdc14 phosphatase (Li et al., 2000) resulting in the accumulation of p53 in the nucleus. However, a recent study suggests that phosphorylation of Ser315 is induced after exposure to UV irradiation and is associated with increased *trans*-activation of target genes (Blaydes et al., 2001). Thus, the mechanism by which roscovitine induces the accumulation of p53 may be independent of its inhibitory activity against Cdk2/Cdc2-induced phosphorylation of the Ser315 site of p53.

An alternative mechanism for the induction of p53 in roscovitine-treated cells may be associated with the effects rosco-

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ABBREVIATIONS: roscovitine, 2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine; TCA, trichloroacetic acid; Cdk, cyclin-dependent kinase; PBS, phosphate-buffered saline; PBSBT, phosphate-buffered saline with bovine serum albumin and Tween-20; CTD, carboxyl terminal domain; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; H7, 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine; Lys382, Lys382 acetyl-specific antibody.

vitine may have on transcription by inhibiting Cdk7. We have previously shown that blockage of RNA polymerase II triggers the induction of both p53 and apoptosis in human cells (Ljungman and Zhang, 1996; McKay et al., 1998; Ljungman et al., 1999). Cdk7 is part of the general transcription factor TFIIH and is thought to be involved in the transition from the initiating stage to the elongating stage of transcription by phosphorylating the carboxyl-terminal domain (CTD) of RNA polymerase II (Feaver et al., 1994; Akoulitchev et al., 1995; Serizawa et al., 1995; Shiekhattar et al., 1995). TFIIH has also been shown to be involved in transcription initiation by melting promoter DNA (Kim et al., 2000) and in transcription elongation (Yankulov et al., 1996). Thus, the inhibition of Cdk7 by roscovitine may have consequences for transcription.

In this study, we investigated whether the induction of p53 by roscovitine may be related to its effects on transcription. We show that roscovitine blocks the phosphorylation of the CTD of RNA polymerase II and inhibits mRNA synthesis in human cells. Thus, the growth suppressive activity of roscovitine may be a combination of Cdk inactivation and the accumulation of p53 and induction of apoptosis by its inhibitory effect on transcription.

Experimental Procedures

Cell Culture. Human neonatal diploid fibroblasts, a gift from Dr. Mary Davis (University of Michigan, Ann Arbor, MI) were grown as monolayers in culture dishes or on microscope coverslips in minimum essential medium supplemented with 10% fetal bovine serum, 2× vitamins, 2× amino acids, and 1× antibiotics. The human colon carcinoma cell line HCT116 was grown as a monolayer in RPMI medium supplemented with 10% fetal bovine serum and 1× antibiotics. Cells were seeded 2 days before each experiment. For the UV-irradiation experiments, cells were irradiated with 20 J/m^2 UV light (254 nm) at room temperature at a fluency of 0.6 J/m^2/s (UVX radiometer, UVP, Inc., Upland, CA) and then incubated at 37°C for 2 or 24 h. For the chemical treatments, a 10 mM stock solution of roscovitine (Calbiochem, La Jolla, CA) in dimethyl sulfoxide and a 50 mM stock solution of 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine (Sigma, St. Louis, MO) in water were added to culture media in concentrations and for periods indicated.

Measurements of Nascent RNA Synthesis. Diploid human fibroblasts were prelabeled with [^{14}C]thymidine by addition of 185 Bq/ml to growth medium 2 days before the experiments. Nascent RNA was labeled for 30 min by adding [^{3}H]uridine (1.5×10^6 Bq/ml). Cells were then rinsed twice in ice-cold PBS, detached by scraping and collected by centrifugation. Poly(A)-enriched RNA was isolated from cell lysates using the Straight A's mRNA Isolation System (Novagen, Madison WI). Total nascent RNA synthesis was measured by precipitating cell lysates with an equal volume of 10% ice-cold TCA. The samples were kept on ice for 30 min and the TCA insoluble material was collected on filters (GF/A; Whatman Inc., Newton Center, MA). The filters were washed with 5 × 1 ml of 5% TCA, 5 × 1 ml dH_2O and 2 × 1 ml of 95% ethanol and then dried under a heating lamp. The ^{3}H and ^{14}C counts present on the filters were counted in a scintillator using a dual counting program. Relative total RNA synthesis and poly(A)RNA synthesis was then determined by calculating the ratio of ^{3}H/^{14}C for each sample and comparing it with the ratio from an untreated control sample. The data are presented as the percentage of the ^{3}H/^{14}C ratio for each treatment compared with this value determined from unirradiated control cells.

Western Blotting. Cells were rinsed in PBS, detached by scraping and collected by centrifugation. Cells were lysed by boiling them in a loading buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromphenol blue, and 62.5 mM Tris, pH 6.8). Samples were subsequently sonicated for 12 s using a microtip (Misonix, Inc., Farmingdale, NY). Protein concentration was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) and approximately 30 μg of protein was loaded per lane. For the experiments analyzing RNA polymerase II, the cell lysates were run on a 6% polyacrylamide gel, and the analysis of p53 proteins were performed using a 12% gel. Proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) overnight at 4°C. The antibodies used were anti-polIILS (N-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA) anti-Ser15 phosphospecific antibody (Ser15, Ab-3; Cell Signaling Technology, Cambridge, MA), anti-Lys382 acetyl-specific antibody (Lys382), and anti-p53 antibody (Ab-2; Oncogene Research Products, Boston, MA). The enhanced chemiluminescent Super Signal CL-HRP Substrate System (Pierce, Rockford, IL) was used to visualize the proteins on X-ray film. The quality of total protein transfer was assessed by staining blots with Coomassie Brilliant Blue after exposure of the membranes to X-ray film. Images were scanned using a flatbed scanner.

Immunofluorescence Microscopy. Human fibroblasts were grown on cover slips and were either mock-treated or treated with 5, 25, or 50 μM roscovitine for 24 h. Cells were then fixed and stained as described previously (Chen et al., 2000; McKay et al., 2001). In short, cells were rinsed in PBS, fixed (50% methanol/50% acetone) and stored at −20°C for about 1 h. The coverslips were then rinsed twice in PBS and once in PBSBT (5 g of bovine serum albumin and 500 μl of Tween-20/l of PBS) before the cells were incubated with the mouse monoclonal anti-p53 antibody 1801 (a gift from Dr. Jiayuh Lin, University of Michigan, Ann Arbor, MI) for 1 h. The samples were then rinsed three times for 5 min with PBSBT before being incubated for 1 h in the dark with 100 μl of a secondary fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (Sigma, St Louis, MO) 1:1000 dilution in PBSBT. Coverslips were rinsed three times in PBSBT and were then mounted on microscope slides in one drop of Vectashield (Vector Laboratories, Inc., Burlingame, CA) and viewed using a fluorescent microscope (Eclipse E600; Nikon, Melville, NY). Images were captured using a digital camera (i308; MicroImage Video Systems, Bechtelsville, PA) and analyzed on a Macintosh computer (Apple, Cupertino, CA) using Adobe PhotoShop (Adobe Systems, Mountain View, CA).

Results

Roscovitine Is a Potent Inhibitor of RNA Synthesis. It has been shown that roscovitine can inhibit Cdk7 (Hajduch et al., 1999), which is a component of the transcription factor TFIIH. Because Cdk7 can phosphorylate the CTD of RNA polymerase II and thus regulate transcription (Feaver et al., 1994; Akoulitchev et al., 1995; Serizawa et al., 1995; Shiekhattar et al., 1995), we investigated whether roscovitine may inhibit RNA synthesis. In fact, previous studies have suggested that roscovitine can affect RNA synthesis in mollusks (Sankrithi and Eskin, 1999) and transcription of viral genes (Schang et al., 2000) in the dose range of 10 to 100 μM.

Here we show that roscovitine rapidly inhibited transcription in both human fibroblasts and HCT116 colon cancer cells (Fig. 1A). A dose of 5 to 50 μM roscovitine reduced mRNA to below 35% in both cell lines within 2 h of treatment. Interestingly, the inhibition of mRNA synthesis was not complete even at higher doses leaving 20 to 30% mRNA synthesis uninhibited. Close examination of the effect of roscovitine on RNA synthesis at doses between 0 and 5 μM revealed that RNA synthesis was affected at doses as low as 1 and 2 μM (Fig. 1B). Because the IC_{50} value for Cdk7 inhibition is about 1.4 μM (Hajduch et al., 1999), it is possible that the effect

roscovitine has on transcription in this dose range is caused by inhibition of Cdk7.

Roscovitine Inhibits the Phosphorylation of the Carboxyl Terminal Domain of RNA Polymerase II. We next investigated whether roscovitine may interfere with the phosphorylation of the CTD of RNA polymerase II. Cdk7 and Cdk9 have been found to phosphorylate multiple sites of the CTD of RNA polymerase (Price, 2000). These modifications are required for promoter escape and for the assembly of RNA processing factors on the CTD of the elongating RNA polymerase (Hirose and Manley, 2000; Proudfoot, 2000). We have previously shown that the unphosphorylated IIa form of the largest subunit of RNA polymerase II, which is the form that is used for the assembly of the transcription initiation complex, rapidly disappears in cells exposed to UV light (McKay et al., 2001). However, we found that the IIa form of RNA polymerase II could be protected after UV irradiation when the CTD kinase inhibitors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine (H7) were present after UV irradiation (McKay et al., 2001). Thus, the loss of the IIa form after UV irradiation is most likely caused by continued initiation and CTD phosphorylation, whereas the elongating form (IIo) becomes trapped at DNA lesions and is therefore unable to "recycle" to replenish the pool of unphosphorylated IIa after completion of transcription.

In agreement with our previous study (McKay et al., 2001), we here show that UV-irradiation causes the loss of the unphosphorylated IIa form of the largest subunit of RNA polymerase II and that treatment with the CTD kinase inhibitor H7 blocked this loss of the IIa form (Fig. 2). Moreover, we show that treatment with roscovitine also blocked the loss of the IIa form of the RNA polymerase after UV irradiation. These results strongly suggest that roscovitine have activities similar to the CTD kinase inhibitor H7.

Roscovitine Induces Nuclear Accumulation of p53 That Is not Modified at Ser15 or Lys382. We next assessed the cellular localization of p53 after roscovitine treatment in diploid human fibroblasts and HCT116 cells. In support of a previous report (David-Pfeuty, 1999), our results show that treatment of cells with 5 to 50 μM roscovitine for 16 h induces nuclear accumulation of p53 in primary human fibroblasts (Fig. 3A) and in HCT116 cells (data not shown).

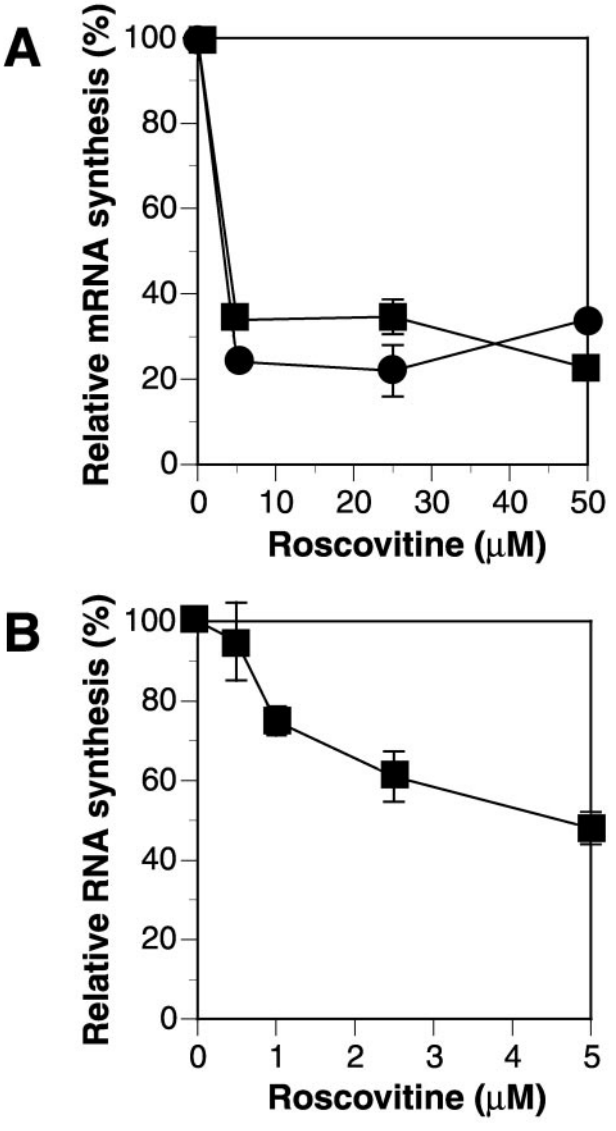

Fig. 1. Roscovitine is a potent inhibitor of RNA synthesis. A, cells were mock-treated or incubated with 5 to 50 μM roscovitine for 2 h. Nascent RNA was labeled with [^{3}H]uridine for 30 min and mRNA was isolated and counted. The data represents the average of duplicate samples; error bars show S.E.M. ●, normal fibroblasts; ■, HCT116 cells. B, Diploid fibroblasts were incubated for 2 h with indicated doses of roscovitine. Nascent total RNA synthesis was then determined. The data represents the average of duplicate samples; error bars show S.E.M.

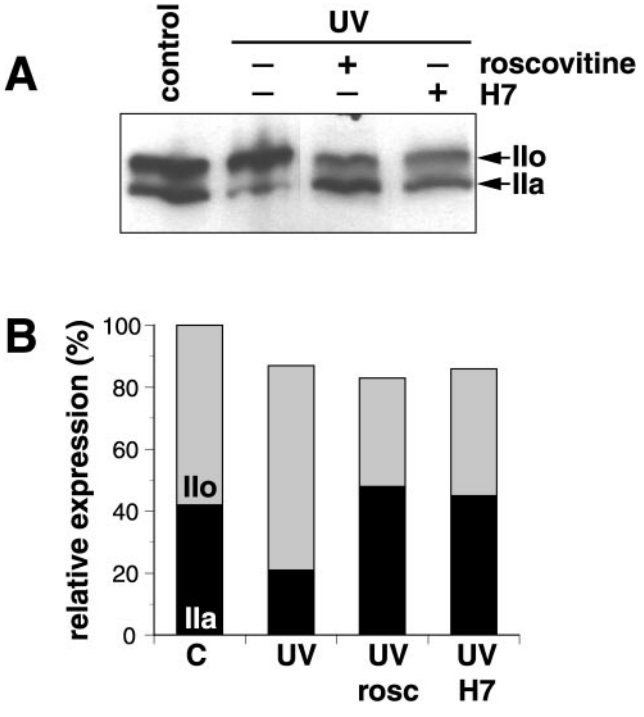

Fig. 2. Roscovitine inhibits the phosphorylation of the CTD of RNA polymerase II after UV-irradiation. A, HCT116 cells were mock-treated or treated for 30 min with either 50 μM roscovitine or 50 μM H7. The media were then removed and the cells were irradiated with 30 J/m^2 followed by incubation in the same media (with the drugs) for 2 h. The level of the unphosphorylated IIa and phosphorylated IIo forms of the largest subunit of RNA polymerase were determined by Western blot. B, quantification of the bands in Fig. 2A was performed using National Institutes of Health Image 1.62 software. The intensities of the IIo and IIa bands were expressed as percentages of the sum of the bands in the control lane. The data is presented as a composite bar diagram with the values of the IIo band on top (gray) and IIa at the bottom (black).

Interestingly, not all cells induced p53 accumulation at lower doses. However, the cells that did accumulate p53 did so to a level similar to that after exposures to high doses. Thus, the induction of p53 accumulation seems to be an "all or none" response after treatment with roscovitine.

We next examined whether the nuclear accumulation of p53 was accompanied by specific modifications of the p53 protein. The MDM2 protein is thought to direct the nuclear export and degradation of p53. By phosphorylating p53 at some specific residues in the N terminus of p53, including Ser15, the interaction between p53 and MDM2 is inhibited (Shieh et al., 1997; Siliciano et al., 1997; Chehab et al., 1999; Unger et al., 1999). Furthermore, the Lys382 residue of p53 has been shown to become acetylated after exposure to UV light or ionizing radiation (Sakaguchi et al., 1998) as well as after inhibition of RNA polymerase II elongation (Ljungman et al., 2001). The acetylation of Lys382 is associated with enhancement of the sequence-specific DNA binding of p53 (Sakaguchi et al., 1998). We found that in contrast to UV light, the p53 proteins that accumulated after roscovitine treatment were not phosphorylated at the Ser15 site (Fig. 3B). Furthermore, acetylation of Lys382 was apparent after UV irradiation but not after roscovitine treatment (Fig. 3C). Thus, although p53 accumulated in the nucleus after exposure to either roscovitine or UV light, modifications of p53 at the Ser15 or Lys382 sites were associated with UV irradiation but not with roscovitine treatment.

Discussion

In this study, we show that roscovitine is a potent inhibitor of both mRNA and total RNA synthesis in human skin fibroblasts and colon carcinoma cells (Fig. 1). Inhibition of RNA synthesis was observed at doses as low as 1 to 2 μM roscovitine. This is in the dose range at which roscovitine has been shown to inhibit Cdk7 (Hajduch et al., 1999), a component of TFIIH. Because Cdk7, in addition to Cdk9, regulate the phosphorylation of the CTD of the largest subunit of RNA polymerase II, it is possible that roscovitine inhibits RNA synthesis by attenuating CTD phosphorylation. In support for this hypothesis is our finding that roscovitine suppressed CTD phosphorylation after UV irradiation (Fig. 2). Interestingly, a 20 to 30% fraction of full-length mRNA synthesis seemed to be immune to the inhibitory affects of roscovitine even at high doses (Fig. 1A), suggesting that a subset of genes may be transcribed in a roscovitine-insensitive and perhaps Cdk7-independent manner.

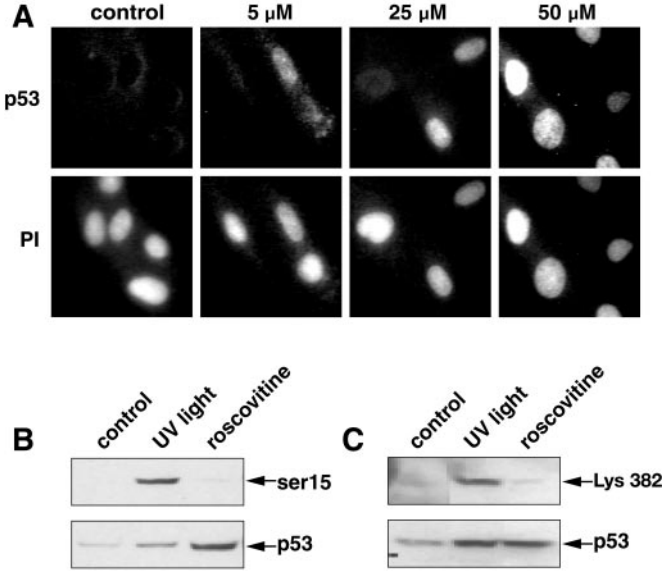

Fig. 3. Roscovitine induces nuclear accumulation of p53 proteins that are not modified at either the Ser15 or Lys382 sites of p53. A, diploid human fibroblasts were grown on coverslips and treated with different concentrations of roscovitine. After 16 h, the cells were fixed and p53 localization was analyzed using immunocytochemistry with mouse monoclonal anti-p53 antibodies (top). The cells were counter-stained with propidium iodide to stain DNA (bottom). B, diploid human fibroblasts were irradiated with 20 J/m^2 of UV light (254 nm) or exposed to 50 μM roscovitine. After 2 h, cells were harvested and the levels of Ser15-modified p53 (top lanes) and total amounts of p53 (lower lanes) were analyzed by Western blot. C, as in A, but cells were harvested 24 h after UV-irradiation or after 24 h of roscovitine treatment and the acetylation status of the Lys382 site of p53 was examined.

In agreement with a previous study (David-Pfeuty, 1999), we show that roscovitine induces the nuclear accumulation of p53 (Fig. 3). The mechanism for p53 induction may be related to the inhibition of Cdc2 and Cdk2-mediated phosphorylation of the Ser315 site of p53 (David-Pfeuty, 1999; Ljungman, 2000). However, in this study, we present an alternative possibility that p53 may accumulate in the roscovitine-treated cells because of inhibition of transcription. Inhibition of RNA polymerase II-mediated transcription has been shown to be closely linked to the induction of p53 (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; McKay et al., 1998; Ljungman et al., 1999; McKay and Ljungman, 1999). Thus, we propose that roscovitine may trigger p53 accumulation by inhibiting transcription. However, the mechanism by which roscovitine-induced inhibition of mRNA synthesis triggers p53 is not clear. The absence of Ser15 and Lys382 modifications of the roscovitine-induced p53 proteins suggests that it may involve a passive mechanism, such as inhibition of MDM2 expression (Blattner et al., 1999; Ashcroft et al., 2000). Alternatively, inhibition of transcription may interfere with the nuclear export machinery (Groulx et al., 2000).

In summary, we suggest that roscovitine induces growth suppression of human cells by inhibition of Cdk activity and by blocking the phosphorylation of the CTD of RNA polymerase II. Thus, roscovitine may act in a similar manner as DRB and H7, which are potent inhibitors of the CTD kinases Cdk7 and Cdk9 (Dubois et al., 1994; Marshall et al., 1996). Further support that roscovitine may share cellular activities with DRB and H7 comes from our finding that the accumulation of p53 after roscovitine treatment was not associated with modifications at Ser15 or Lys382 (Ljungman et al., 2001). In addition, the nucleolar fragmentation previously observed after roscovitine treatment (David-Pfeuty, 1999) also occurs after DRB treatment and is thought to be related to inhibition of RNA polymerase II-mediated transcription (Haaf and Ward, 1996). Because roscovitine has recently attracted attention as a potential anticancer agent (Yakisich et al., 1999a; Buolamwini, 2000; Edamatsu et al., 2000), our findings that roscovitine inhibits RNA synthesis in human cells should be helpful for the understanding of the molecular and cellular mechanism of action of this drug.

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Address correspondence to: Dr. Mats Ljungman, Department of Radiation Oncology, Division of Cancer Biology, University of Michigan Comprehensive Cancer Center, 4306 CCGC, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0936. E-mail: ljungman@umich.edu