Roscovitine-induced up-regulation of p53AIP1 protein precedes the onset of apoptosis in human MCF-7 breast cancer cells

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Abstract
We reported recently that roscovitine arrested human MCF-7 cancer cells at G2-M phase of the cell cycle and concomitantly induced apoptosis. After roscovitine treatment, the level of wild-type p53 protein strongly increased and p53 was accumulated in the nucleus. Here, we raised the question of which pathway would be involved in roscovitine-induced apoptosis in MCF-7 cells, which are known to be caspase-3-deficient, and whether roscovitine-mediated activation of p53 protein might positively affect the execution of cell death. Roscovitine induced a depolarization of mitochondrial potential beginning at 6 hours posttreatment as evidenced by changes in J-aggregate formation and release of the mitochondrial proteins cytochrome c and apoptosis-inducing factor. Interestingly, roscovitine stimulated a site-specific phosphorylation of wild-type p53 protein in a time-dependent manner. p53 protein was specifically phosphorylated at Ser46. P-Ser46-activated wild-type p53 tumor suppressor up-regulated p53AIP1 protein, its downstream target known to mediate the depolarization of mitochondria. The onset of phosphorylation of p53 at Ser46 preceded the up-regulation of p53AIP1 protein and the depolarization of mitochondrial potential. We compared the kinetics of roscovitine-mediated p53 activation between caspase-3-deficient parental MCF-7 cells and cells reconstituted with caspase-3. The kinetics and the extent of p53 protein activation in caspase-3-proficient cells differed from those observed in caspase-3-deficient parental cells. Remarkably, roscovitine failed to induce phosphorylation at Ser46 in caspase-3-reconstituted MCF-7 cells. Our results indicate that, depending on the status of caspase-3 in MCF-7 cells, different apoptotic pathways were initialized.

Introduction
The tumor suppressor p53 controls multiple downstream targets that regulate variable cellular outcomes such as transient or permanent growth arrest, apoptosis, and cellular senescence (for reviews, see refs. 1, 2). The wild-type p53 protein affects the progression of the cell cycle at distinct phases and supervises the essential checkpoints at G1-S and G2-M (3–5). In unstrained cells, the wild-type p53 protein is maintained in low concentrations. The expression and intracellular distribution of p53 varies throughout the cell cycle. Wild-type p53 is predominantly nuclear in G1 and largely cytoplasmic during S and G2 (6). This distribution pattern of tumor suppressor p53 protein is consistent with its physiologic function. However, in response to a variety of stress stimuli, p53 becomes stabilized and activated p53 protein accumulates in the nucleus, thereby regulating the transcriptional activity of its downstream effectors. Different genotoxic stress stimuli such as UV, IR, or chemical DNA damaging agents are known to up-regulate wild-type p53 protein. Remarkably, several nongenotoxic stimuli such as generation of reactive oxygen species or disturbance of the nucleotide pool have been reported to target cellular p53 response. Interestingly, the newly developed synthetic inhibitors of cyclin-dependent kinases (7–9), recognized as potent anticancer drugs (10, 11), have been found to stabilize p53 protein (12–14). Flavopiridol and roscovitine, highly specific cyclin-dependent kinase inhibitors, are able to increase wild-type p53 protein levels (15–17). The flavopiridol-mediated inhibition of transcription resulting in down-regulation of MDM-2 (13), a negative regulator of p53 protein, essentially contributes to the stabilization of wild-type p53 protein. It seems that lengthening of the p53 half-life in roscovitine-treated MCF-7 cells is attributable to the same mechanism (18).

Human breast cancer MCF-7 cells are relatively resistant to the action of a variety of conventional cytostatic drugs (19). The decreased efficacy of some anticancer drugs towards MCF-7 cells seems to be attributable to the lack of caspase-3 expression owing to a 47 bp deletion within exon 3 of the caspase-3 gene (20). Therefore, the treatment of MCF-7 cells by alternative drugs exhibiting low direct cytotoxicity on the one side and concomitant targeting p53 response on the other side could potentiate the therapeutic efficacy by induction of apoptotic steps independent of caspase-3 activity. The p53 tumor suppressor is known not only to initiate apoptosis (21, 22) but also to affect its execution at...
different stages (23). The stimulated p53 protein enhances the activity of distinct proapoptotic genes such as Bax, Apaf-1, Peg3, PUMA and caspase-9 and is also able to repress some antiapoptotic genes such as Bcl-2. Recently, a new p53-dependent proapoptotic gene, p53-apoptosis inducing protein-1 (p53AIP-1) was identified (24). p53AIP-1, a component of the mitochondrial membrane, has been found to be regulated by p53 protein in a highly specific way (25). Only wild-type p53 protein phosphorylated at Ser46 was transcriptionally competent to induce p53AIP-1 protein (24). It has been shown that upon severe DNA damage, Ser46 on p53 was phosphorylated, resulting in the induction of p53AIP-1 protein followed by depolarization of mitochondrial membrane and sequentially by release of distinct mitochondrial proteins such as cytochrome c and apoptosis inducing factor (AIF; refs. 24, 25). Ectopically expressed p53AIP-1 protein, which was localized in mitochondria, led to apoptosis through dissipation of the mitochondrial potential (24).

Recently, we observed that roscovitine induced a strong cell cycle arrest of the human breast cancer cells MCF-7 beginning at 6 hours after onset of the treatment (17). Inhibition of DNA replication and accumulation of G2-M–arrested cells coincided with a marked up-regulation of p53 protein and with the appearance of a few Annexin-V–positive cells. The main wave of apoptosis was observed after exposure of MCF-7 cells to roscovitine for 24 hours.

In the present work, we raised the question by which pathway roscovitine initiates apoptosis in human breast cancer MCF-7 cells. We observed the decrease of DeltaPsim of the mitochondrial membrane beginning at 6 hours as detected by the stepwise loss of the formation of J-aggregates and by the release of the distinct mitochondrial proteins such as cytochrome c and AIF. We also asked whether the roscovitine-stimulated wild-type p53 is functionally linked to ongoing apoptosis. Roscovitine-induced phosphorylation of p53 protein at Ser46. The strong site-specific phosphorylation of p53 protein occurred after 4 hours of roscovitine treatment and preceded the onset of mitochondria depolarization by 2 hours. The P-Ser46-activated tumor suppressor protein became transcriptionally competent and induced up-regulation of p53AIP-1 protein. Finally, we compared the kinetics of roscovitine-mediated p53 activation between caspase-3-deficient parental MCF-7 cells and cells reconstituted with caspase-3. The kinetics and the extent of p53 protein activation in caspase-3-proficient cells differed from those observed in caspase-3-deficient parental cells. Remarkably, roscovitine failed to induce phosphorylation at Ser46 in caspase-3-reconstituted MCF-7 cells. Our results indicate that depending on the status of caspase-3 in MCF-7 cells, different apoptotic pathways were initialized.

Materials and Methods

Cells

Human breast carcinoma MCF-cells were grown as a monolayer in Dulbecco’s medium without phenol red, supplemented with 10% FCS at 37°C in an atmosphere of 8% CO2. In some experiments, the caspase-3-proficient MCF-7.3.28 cell line or cells transfected with control vector (20) or normal human MRC-5 fibroblasts were used (26) and were maintained as described earlier. Cells were grown to 60% to 70% confluence and then treated with roscovitine in a final concentration ranging from 1 to 20 μmol/L for indicated periods of time. Roscovitine was dissolved as a stock solution in DMSO and stored at −20°C until use.

Antibodies

We used the following antibodies: monoclonal anti-p53 antibody DO-1, a kind gift from Dr. B. Voješek (Masaryk Memorial Cancer Institute, Brno, Czech Republic), anti-MDM-2 (2A10) antibodies, a kind gift from Dr. A. Levine, (Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, New Brunswick, NJ) monoclonal anti-PARP-1 antibodies (C-2-10) from Dr. G. Poirier (Laval University, Quebec, Canada). Polyclonal anti-p53 antibodies CM-1 were obtained from Novocastra (Newcastle-upon-Tyne, United Kingdom). Monoclonal anti-AIF antibody (E-1), anti-MDM-2 (D-12) antibodies and polyclonal anti-p53AIP1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-cytochrome c antibody (clone 7H8.2C12) was from BD PharMingen International (San Diego, CA), monoclonal anti-MCM7 (clone DCS141.2) and anti-proliferating cell nuclear antigen (PCNA; PC-10) antibodies were from Oncogene Research Products (Cambridge, MA). Polyclonal anti-caspase-3 antibodies were from Dako (Glostrup, Denmark), anti-p21Waf1 antibodies (clone DCS 60) and polyclonal anti-phospho-Ser46-p53 were from New England Biolabs (Beverly, MA). Rat monoclonal antibodies to apoptotic protease-activating factor-1 (APAF-1; clone 2E12) were from Chemicon International, Inc. (Temecula, CA), antibodies against Smac/Diablo (clone 10G7) were from Alexis Biochemicals Corp. (San Diego, CA), anti-ran antibodies (clone 20) were from Transduction Laboratories (Lexington, KY), and polyclonal anti-HtrA2/Omi antibodies were from R&D Systems, Inc. (Minneapolis, MN). Monoclonal anti-actin (clone C4) antibodies were from ICN Biochemicals (Aurora, OH). Appropriate secondary antibodies linked to horseradish peroxidase or fluorochromes (Cy-2 and Cy-3) were from Amersham International (Little Chalfont, Buckinghamshire, United Kingdom). The secondary antibodies, especially those used for double immunostaining, were prepared from monospecific antiserum by immunoaffinity chromatography followed by multiple solid phase adsorptions to eliminate possible cross-reactivity.

Immunohistochemical Staining

For microscopic investigations, cells were plated on slides in chambers and appropriately cultivated. After treatment for the indicated times, cells were washed thrice in PBS, fixed in ice-cold methanol-acetone (3:2) mixture and stained (27). For visualization of nuclei, cells were sequentially stained with 4,6-diamidino-2-phenylindole dissolved in PBS to a final concentration of 1 μg/mL.

Measurement of DNA of Single Cells by Flow Cytometry

The measurement of DNA content was done by flow cytometric analysis based on a slightly modified method.
(28) described previously by Vindelov et al. (29). The cells were detached from the substratum by trypsinization, then all cells were harvested by centrifugation and washed in PBS. Aliquots of $1 \times 10^6$ 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 hours incubation at +4°C in the dark.

**Quantitative Analysis of the Mitochondrial Membrane Potential by Flow Cytometry**

Mitochondrial depolarization was monitored using the cationic carbocyanine dye JC-1 (Molecular Probes Inc. Eugene, OR, USA). Control and roscovitine treated cells were trypsinized, washed and incubated with the dye at a final concentration of 10 μmol/L for 5 minutes followed by extensive washings in PBS and immediate two-color analysis under fluorescence microscopy and by fluorescence-activated cell sorting (22). Alternatively, cells in Petri dishes were washed with PBS and directly incubated with JC-1. After washing, samples were inspected under fluorescence microscopy using a bandpass filter (detects fluorescein and rhodamine). JC-1 exists as red fluorescent aggregates (excitation/emission at 488–570 nm) in intact cells due to potential-driven mitochondrial accumulation and aggregation. Upon mitochondrial depolarization, it occurs as green fluorescent monomers (excitation/emission at 488–530 nm).

**Quantification of Cellular p53 Protein by ELISA**

The cellular level of total p53 protein in cell extracts was measured using the p53 ELISA kit from Biosource (Biosource International, Inc., Camarillo, CA). Cells were extracted using a buffer recommended by the supplier. The determination was done according to the protocol recommended by the supplier. Absorbance for p53 standards and specimens was read at 450 nm. The p53 concentration determined in cell extracts was calculated from the standard curve and was normalized per microgram of protein.

**Subcellular Fractionation of Cells**

In experiments designed to examine the release of distinct proteins from mitochondria during the execution of apoptosis, buffer containing 250 mmol/L sucrose and a low concentration of digitonin was used for cell fractionation (30).

**Electrophoretic Separation of Proteins and Immunoblotting**

Total cellular proteins or proteins of the distinct subcellular fractions dissolved in SDS sample buffer were separated on 10% or 15% SDS slab gels, transferred electrophoretically onto polyvinylidene difluoride (Amersham International) and immunoblotted as previously described (31, 32). Equal protein loading was confirmed by Ponceau S staining. To determine the phosphorylation status of selected proteins, antibodies recognizing site-specific phosphorylated proteins were diluted to a final concentration of 1:1,000 in 1% bovine serum albumin in Tris-saline-Tween 20 buffer (33). In some cases, blots were used for sequential incubations.

**Results**

**Roscovitine Induces Changes of the Membrane Potential of Mitochondria**

To assess the effect of roscovitine treatment on the integrity of the mitochondrial membrane, we did two distinct approaches. First, we monitored the membrane potential using an electrochromic dye JC-1 (34), and secondly, we examined the release of distinct mitochondrial proteins into the cytosol. JC-1, a carbocyanine with a delocalized positive charge, redistributes between compartments according to the membrane potential. The membrane potential of energized mitochondria (negative inside) promotes an uptake of JC-1 into the matrix and subsequent formation of J-aggregates. Untreated controls and cells treated with 20 μmol/L roscovitine for indicated periods of time were exposed to JC-1 directly in Petri dishes or were detached from the substratum by limited trypsinization and then incubated with JC-1. After thorough washing, cells were immediately analyzed. Two-color analysis was done under fluorescence microscopy and the fluorescence was additionally quantified by fluorescence-activated cell sorting. As shown in Fig. 1 (top) almost all untreated control cells formed J-aggregates. After treatment with 20 μmol/L roscovitine for 6 hours, several cells failed to complex JC-1 in mitochondria and monomeric dye generating green fluorescent signals accumulated in the cytosol. The quantification by fluorescence-activated cell sorting revealed the appearance of about 10% green fluorescent cells after 10 hours (Fig. 1, bottom). After longer exposure of cells to roscovitine, the frequency of green fluorescent cells increased and at 15 hours, reached about 50% of gated MCF-7 cells. We parallelly analyzed the caspase-3-proficient MCF-7 cells. Surprisingly, the reconstitution of human breast carcinoma MCF-7 cells with caspase-3 did not enhance the effect of roscovitine on the depolarization of mitochondria.

**Accumulation of Mitochondrial Proteins in Cytosol upon Roscovitine Treatment**

In a second approach, we determined the accumulation of the mitochondrial proteins in the cytosol. To avoid any artificial damage of mitochondrial membrane during the isolation procedure, we gently fractionated cells by a method described by Fiskum et al. (30). The cytosol sample prepared from control MCF-7 cells did not contain mitochondrial proteins such as cytochrome c or AIF (Fig. 2). However, these proteins were detected in cytosol isolated from roscovitine-treated MCF-7 cells. Moreover, two other proteins, a second mitochondria-derived activator of caspase/direct inhibitors of apoptosis (IAP) binding protein with low isoelectric point (Smac/Diablo) and a processed serine protease HtrA2/Omi were released from mitochondria during roscovitine treatment (Fig. 2). The protein band at about 50 kDa corresponding to the newly synthesized serine protease Omi was present in all cytosol samples. However, its 37 kDa truncated form generated after transport into mitochondria...
dria was accumulated in cytosol beginning at 10 hours posttreatment. Finally, the level of APAF-1 protein markedly increased in the cytosol after roscovitine treatment for 10 hours (Fig. 2). APAF-1 is a cytosolic protein that rests in a latent state until bound to cytochrome c. This protein is commonly released from the mitochondria during apoptosis. Accumulation of AIF in cytosol beginning at 6 hours was accompanied by diminution of the AIF levels in isolated mitochondria (Fig. 2).

**Figure 1.** Disruption of the mitochondrial transmembrane potential in human breast carcinoma MCF-7 cells treated with roscovitine. A, loss of formation of J-aggregates after roscovitine treatment. Controls and cells treated with 20 μmol/L roscovitine for 6 h were stained in situ with JC-1 and immediately analyzed by fluorescence microscopy using a bandpass filter (which detects FITC and rhodamine). Cells present in the field were visualized by Hoffman modulation contrast (HMC). In untreated control, a mitotic cell (M) is indicated by the white arrow. B, two-color analysis by flow cytometry using the FITC channel for green monomers (Ex/Em = 510/527) and the PI channel for red aggregates (Ex/Em = 585/590). Numbers of green fluorescent cells were included. Circles, the population of cells that lost the capability to aggregate JC-1 dye. Parental MCF-7 and caspase-3 reconstituted MCF-7.3.28 cells were analyzed.

**Caspase-3 Reconstitution Did Not Increase the Apoptotic Chromatin Fragmentation in MCF-7 Cells after Roscovitine**

To assess the effect of caspase-3 reconstitution on the execution of roscovitine-induced apoptosis, we did the cytometric analysis of propidium iodide–stained cells. We analyzed parental MCF-7 cells and caspase-3-reconstituted MCF-7.3.28 cells (20). For comparison, MCF-7.0.3 cells transfected with control vector were included. Flow cytometric assessment of an apoptotic cell population
based on DNA content is made possible by loss of fragmented DNA after cell permeabilization. As illustrated in Fig. 3A, no sub-G₁ cell population could be detected in parental MCF-7 cells after roscovitine treatment. Surprisingly, caspase-3 reconstitution of MCF-7 did not induce chromatin fragmentation upon roscovitine exposure. However, the effect of caspase-3 reconstitution became evident after continuous roscovitine treatment for 24 hours and postincubation for 24 hours in a drug-free medium. The DNA profile obtained from caspase-3-proficient cells showed, in contrast to parental cells, changes indicative of apoptotic chromatin breakdown (Fig. 3A).

**Prolonged Cell Cycle Arrest in Roscovitine-Treated MCF-7 Cells**

The analysis of DNA histograms revealed that roscovitine very rapidly arrested MCF-7 cells in G₂ phase of the cell cycle. At 6 hours, about 20% of cell population was in G₂ and the frequency of this population increased by 10% after a further 18 hours. Interestingly, roscovitine-induced G₂ arrest in MCF-7 cells was prolonged because the frequency of the population of G₂ arrested cells remained almost unchanged after postincubation for 24 hours in a drug-free medium. In contrast to a rapid and strong inhibition of cell cycle in parental cells exposed to roscovitine, caspase-3-proficient MCF-7 cells responded much weaker to the drug (Fig. 3A). A comparison of the changes of the G₂/G₁ ratio between caspase-3-deficient and -proficient cells in the course of roscovitine treatment illustrates very well the difference in the effect of roscovitine on the cell cycle progression (Fig. 3B).

**Kinetics of p53 Activation**

We determined the kinetics of the roscovitine-mediated p53 up-regulation in MCF-7 cells and compared with that observed in normal human fibroblasts MRC-5. In cell lysates obtained from asynchronously growing MCF-7 control cells, p53 protein was barely detectable. However, after roscovitine treatment, its level markedly increased (Fig. 4). The strong p53 up-regulation observed at 6 hours posttreatment persisted for at least a further 20 hours. At this time point, the apoptotic 89 kDa fragment of PARP-1 was generated in MCF-7 cells. Interestingly, the treatment of normal MRC-5 human cells with roscovitine resulted in a weaker p53 response. After continuous exposure of MRC-5 cells for 24 hours, the p53 level decreased. No degradation of PARP-1 could be detected. The sequential incubation of the blot with anti-PCNA antibodies revealed a different proliferative status between human breast carcinoma MCF-7 cells and normal MRC-5 fibroblasts. The basal PCNA level in MCF-7 was much higher than that in MRC-5 cells. Moreover, the roscovitine treatment positively affected PCNA expression in normal MRC-5 cells but not in cancer MCF-7 cells. After roscovitine treatment for 6 and 15 hours, the PCNA level transiently increased.

We additionally quantified the cellular level of total p53 protein in extracts prepared from controls and roscovitine-treated cells by ELISA. The concentration of p53 protein increased about 40-fold in MCF-7 cells after 6 hours of roscovitine treatment (Fig. 4). In caspase-3-proficient MCF-7 cells, the transient p53 increase was markedly weaker. Its level was about 15-fold elevated after 6 hours of roscovitine treatment.

**Extension of p53 Half-Life after Roscovitine Treatment**

In the next step, we determined the stability of p53 protein in controls and cells treated with roscovitine for 15 hours. The relative amounts of p53 protein remaining at various time points after emetine addition were then measured.

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**Figure 2.** Release of mitochondrial proteins into the cytosol. Proteins of the cytosol (S-100 fraction) or of the mitochondria isolated according to Fiskum et al. (30) from untreated controls and cells treated with 20 μmol/L roscovitine for indicated periods of time were resolved on 10% or 15% SDS gels and transferred onto a polyvinylidene difluoride membrane. The blots were incubated with indicated primary antibodies in the appropriate concentration. After incubation with secondary antibodies linked to horseradish peroxidase, immune complexes were detected by chemiluminescence using ECL+. Equal protein loading was confirmed by Ponceau S staining of the membrane and additionally by incubation with anti-actin or anti-ran antibodies. The detection of actin is suitable for control of equal loading of WCL proteins but not of proteins of different subcellular fractions. Due to isolation procedure, cytosol (S-100) and mitochondria are devoid of actin. Ran protein shuttling between cytosol and nucleus seems to be a better indicator of equal protein concentration.
quantified by densitometric measurements of immuno-
blots. As shown in Fig. 5, the intensity of the p53 band in
untreated control cells decreased very rapidly after
addition of emetine and was reduced by 70% after 10
minutes of incubation. However, in roscovitine-treated
cells, the p53 level remained almost unaffected during the
8 hours after incubation. Sequential incubation of the blots
with anti-PARP-1, anti-MCM-7, anti-PCNA antibody, or
with anti-actin antibody revealed that the roscovitine
treatment affected primarily the steady-state of p53 protein,
whereas the stability of long-lived proteins such as PARP-1
or actin remained unchanged. The evaluation of the p53
half-life by linear regression revealed about 40-fold
lengthening of the p53 stability after roscovitine treatment
for 15 hours ($t_{1/2} = 16$ minutes in control cells and $t_{1/2} =
663$ minutes after 15 hours roscovitine).

Site-Specific Phosphorylation of p53 Protein in MCF-7
Cells

The stability of p53 tumor suppressor is known to be
regulated by posttranslational modifications, especially by
phosphorylation. Therefore, we examined the phosphory-
lation status of p53 protein upon roscovitine treatment
using phosphospecific antibodies. As shown in Fig. 6,
roscovitine induced phosphorylation of p53 at Ser46 in

![Figure 3](image_url)

**Figure 3.** Prolonged cell cycle arrest induced in MCF-7 cells by roscovitine. Parental MCF-7 cells transfected with empty control vector (MCF-7.0.3) and
cells transfected with human caspase-3 (MCF-7.3.28) were treated with 20 μmol/L roscovitine for the indicated periods of time. In some cases, after
treatment for 24 hours, medium was changed (MC) and after addition of a fresh drug-free medium, cells were maintained in cell culture for 24 hours. Cells
were harvested and stained with propidium iodide. **A,** DNA histograms depicting a representative experiment done in duplicate prepared using ModFIT
software; **B,** comparison of the roscovitine effect on the distribution of cells in distinct cell cycle phases. **Top,** values of cells in distinct cell cycle phases. **Bottom,** G2/G1 ratio. Values calculated for each sample were normalized to the control.
MCF-7 cells in a time-dependent manner. The modification at Ser46 was detected in whole cell lysates (WCL) as well as in nuclei isolated from MCF-7 cells exposed to roscovitine. Interestingly, the phosphorylated p53 protein was detected in WCL after exposure of cells to roscovitine for 6 hours, whereas in the isolated nuclei, the phosphorylated p53 form appeared at 10 hours. The sequential incubation of the blot with antibody recognizing total p53 protein revealed the strong accumulation of p53 protein in both WCLs and in isolated nuclei. This observation was additionally confirmed by immunostaining (Fig. 7). No P-Ser46-p53 signals could be detected in untreated MCF-7 cells. Staining with polyclonal and monoclonal antibodies recognizing total p53, irrespective of its modifications, revealed a very weak signal in control cells, detected primarily in the cytoplasm. However, after exposure of MCF-7 cells to roscovitine for 6 hours, positive staining was detected in almost all cells. In the majority of cells, p53 phosphorylated at Ser46 appeared in the cytosol and strong signals were detected only in a small number of nuclei. Remarkably, after longer roscovitine treatment, nuclei strongly stained by anti-P-Ser-46 p53 antibodies showed a condensed chromatin (Fig. 7).

Finally, we proved the earliest onset of roscovitine-induced p53 phosphorylation. After exposure of MCF-7 cells to roscovitine for 3 hours only a weak phosphoserine-46 signal was observed (Fig. 8A). A strong phosphorylation of p53 at Ser46 appeared already after exposure of MCF-7 cells to roscovitine for 4 hours (Fig. 8A).

Lack of p53 Phosphorylation at Ser46 in Caspase-3-Proficient MCF-7 Cells
In caspase-3-reconstituted MCF-7 cells, no phosphorylation of p53 protein at Ser46 could be detected (Fig. 6). Exposure of caspase-3-proficient MCF-7 cells to roscovitine resulted in an induction of obviously weaker p53 response. p53 protein was transiently accumulated in the isolated nuclei reaching the highest level at 6 hours. Remarkably, despite the presence of caspase-3 (Fig. 6), roscovitine did not induce degradation of PARP-1 to the 89 kDa fragment either in the WCL or in the nuclei. These results implicate that the reconstitution of MCF-7 cells with caspase-3 alters the extent and the kinetics of p53 activation induced by roscovitine.

Figure 5. Increased stability of p53 protein in roscovitine-treated MCF-7 cells. Untreated MCF-7 cells (control) and cells treated with 20 μmol/L roscovitine for 15 hours were incubated for the indicated periods of time with emetine to block the protein synthesis. The WCLs (30 μg/lane) were resolved on 10% SDS gels. Immunoblotting was done with anti-p53 antibodies DO-1 and sequentially with anti-PARP-1 antibodies, with anti-MCM-7 antibodies, with anti-PCNA antibodies and with anti-actin antibodies. The relative amounts of p53 protein and PCNA remaining at distinct time points after emetine addition were quantified by densitometric analysis of immunoblots. The relative values calculated for samples lysed immediately after addition of emetine (0 hour) were estimated as 100%. The half-lives ($t_{1/2}$) in control and roscovitine-treated cells were calculated by linear regression. Bottom, the relative values of the remaining p53 band normalized to the corresponding values of actin (columns).

Figure 4. Strong induction of p53 protein in human cells exposed to roscovitine. Top, whole cell extracts prepared from human control cells and cells exposed to 20 μmol/L roscovitine were separated on a 10% SDS gel (30 μg/lane). Transfer and immunoblotting conditions as in Fig. 2. Blots were incubated with monoclonal anti-p53 antibodies DO-1 and sequentially with anti-PARP-1 antibodies, anti-MCM-7 antibodies, anti-PCNA and anti-actin antibodies. The partial lack of the 89-kDa band in the third lane is due to air bubble generation and inappropriate protein transfer. Lysate of human cervix carcinoma HTB-31 cells expressing high levels of mutant p53 was loaded in the last lane. Bottom, the concentration of total p53 in cell extracts prepared from control cells and cells treated with 20 μmol/L roscovitine for the indicated periods of time were determined by ELISA. For the standard curve, p53 protein within the range from 0 to 8 ng/mL was used. The assay was done in duplicate according to the manufacturer's procedure.
Induction of p53AIP1 Protein in Roscovitine-Treated MCF-7 Cells

Roscovitine not only elevated the nuclear level of p53 protein but also activated its transcriptional activity. This became evident after monitoring of the levels of p21waf1 and MDM-2 proteins. The low basal level of p21waf1 was elevated by roscovitine at 4 hours and increased further at 8 hours (Fig. 8A). MDM-2, another p53 target, was strongly up-regulated after roscovitine treatment for 6 hours (Fig. 8A). To assess the functional consequences of the p53 activation by site-specific phosphorylation in roscovitine-treated MCF-7 cells, we monitored the levels of p53AIP1, a known transcriptional target of P-Ser46-p53 (24). In untreated controls, p53AIP1 protein was undetectable in WCLs as well as in isolated mitochondria. However, after roscovitine treatment for 6 hours, p53AIP1 appeared in WCLs (data not shown) as well as in subcellular fractions prepared from MCF-7 cells (Fig. 8B and C), but not in samples obtained from caspase-3-reconstituted (MCF-7.3.28) cells (data not shown). Interestingly, the kinetics and the extent of p53AIP1 up-regulation seems to be dependent on the extent of the phosphorylation of Ser46-p53. If a strong P-Ser46-p53 signal was detected at 4 hours after roscovitine administration (Fig. 8A), a more pronounced p53AIP1 protein expression was observed (Fig. 8C). In such a case, p53AIP1 at a high concentration appeared 6 hours in cytosol, and at 15 hours, p53AIP1 was localized exclusively in mitochondria (Fig. 8C).

Discussion

Human breast carcinoma MCF-7 cells generally exhibit a reduced sensitivity to several anticancer drugs (19, 35). The decreased susceptibility of MCF-7 cells to a variety of
chemotherapeutic agents seems to be at least partially a consequence of the misregulation of the apoptotic pathway. MCF-7 cells lack caspase-3 activity due to a 47-bp deletion within exon 3 of the \textit{caspase-3} gene (20). Recent studies showed the increased antiproliferative efficacy of newly developed cyclin-dependent kinase inhibitors on MCF-7 cells (15, 17, 36). It has also been shown that roscovitine, a highly specific inhibitor of cyclin-dependent kinases, strongly blocked the proliferation of MCF-7 cells by negative regulation of the cell cycle progression. Interestingly, synchronized cells responded quite differently to the action of roscovitine compared with naive, randomly growing cells. MCF-7 cells synchronized by the action of nocodazole were primarily arrested in G1 phase of the cell cycle after exposure to roscovitine (15). However, a naive, asynchronous population of MCF-7 cells was inhibited by roscovitine in the progression between G2 and M cell cycle phases (17). A marked increase in the number of G2-arrested cells was accompanied by the diminution of the number of S phase cells (17). Roscovitine seems to be more efficacious on MCF-7 cells than cisplatin, a widely used anticancer drug (37). A comparison of the action of both drugs on the proliferation and cell cycle progression revealed a clearly stronger inhibitory effect of roscovitine (37).

Moreover, the exposure of asynchronously growing MCF-7 cells to roscovitine induced apoptosis (17). The onset of G2-M arrest and the increase of cellular levels of the tumor suppressor protein p53 preceded the beginning of the process of programmed cell death. A few independent lines of evidence showed that roscovitine-induced apoptosis in MCF-7 cells beginning 6 hours after drug administration proceeded slowly and the main wave of apoptosis occurred between 15 and 24 hours (17). The early stages of roscovitine-induced cell death were characterized by loss of phosphatidylserine asymmetry and activation of caspases (17). The translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface detected by Annexin-V binding began at 6 hours after roscovitine treatment and increased after longer treatment. At 6 hours treatment, activation of caspase-7 and the

\textbf{Figure 8.} Induction of site-specific phosphorylation of p53 precedes the transcriptional activation of p53AIP1 protein in roscovitine-treated MCF-7 cells. Total cellular proteins (A, WCLs) or proteins of subcellular fractions (B and C) prepared from MCF-7 cells were resolved on 10% or 15% SDS gels. Proteins immobilized on membranes were incubated with antibodies as indicated. Protein loading was checked by sequential incubation with anti-actin (A and B). Monitoring of actin concentration was not suitable to compare protein loading of subcellular fractions. Therefore, in C, the nonspecific band at about 50 kDa generated by polyclonal and p53AIP1 antibody was shown.
appearance of an 89-kDa fragment of PARP-1 was observed (17). At this time point, chromatin condensation indicative of apoptosis was detected in several nuclei. In the latter phase of apoptosis beginning 12 hours after roscovitine treatment, DNA fragmentation was observed as assessed by terminal nucleotidyl transferase-mediated nick end labeling assay (17). About 15% of cells showed terminal nucleotidyl transferase-mediated nick end labeling positive signals. At first sight, the generation of an 89-kDa degradation product of PARP-1 in cells lacking caspase-3 activity was surprising. However, PARP-1 is cleaved even in the absence of caspase-3 and the activated caspase-7 could be a potential executioner (17, 38, 39).

In our present study, we raised the question of which pathway roscovitine initiates apoptosis in caspase-3-deficient MCF-7 cells, and whether activated p53 protein may facilitate and positively affect the execution of apoptosis. Caspase-3 is an effector caspase that is activated by apical caspases. Recent reports suggest that caspase-3 is essential for procaspase-9 processing and activation of the apoptosome (40). It is also required for membrane blebbing and chromatin fragmentation. There is a body of evidence indicating that caspase-3 plays an important role in both death receptor- and mitochondria-mediated apoptosis. It has been reported by a few groups that reconstitution of caspase-3 sensitizes MCF-7 cells to distinct chemotherapeutic agents (20, 41). Therefore, we additionally addressed the question of whether caspase-3 reconstitution may potentiate the proapoptotic action of roscovitine on MCF-7 cells.

We observed that roscovitine treatment resulted in the depolarization of the mitochondrial potential as evidenced by loss of formation of J-aggregates and release of distinct mitochondrial proteins into the cytosol. The onset of the loss of transmembrane potential at 6 hours correlated with the appearance of mitochondrial proteins such as AIF, cytochrome c, or Smac/Diablo in the cytosol fraction. At 6 and 10 hours after roscovitine administration about 10% of MCF-7 cells have lost the ability to form J-aggregates. The accumulation of AIF protein in the cytosol was accompanied by a decrease of its intramitochondrial level. Beginning at 6 hours after treatment, a strong degradation of PARP-1 was observed. A marked accumulation of the 89 kDa PARP-1 fragment occurred in isolated nuclei. Remarkably, roscovitine strongly increased the level of p53 protein. After 2 hours of roscovitine treatment, the p53 concentration was highly elevated. At 6 hours, the p53 level increased about 40-fold as compared with the untreated control. Roscovitine treatment resulted in the stabilization of p53 protein. The half-life of p53 protein increased about 40-fold after roscovitine treatment for 15 hours. Interestingly, roscovitine stimulated p53 phosphorylation at Ser46. The roscovitine-induced site-specific phosphorylation of p53 is a novel and complementary finding to another observation according to which the nuclear accumulation of p53 in cells treated with roscovitine was not accompanied by modifications at either the Ser15 or Lys382 sites of p53 (14). Onset of p53 up-regulation preceded the site-specific phosphorylation of Ser46-p53 by 1 to 2 hours. Remarkably, the strong signal for P-Ser46 p53 occurred in several nuclei showing condensed chromatin. It implies a close correlation between p53 activation and execution of apoptosis. The transcriptional competence of roscovitine up-regulated p53 was confirmed by the monitoring of p21^waf1 and MDM-2, known p53 downstream targets. The transcriptional activation of p21^waf1 began at 4 hours and preceded the increase of MDM-2 protein observed at 6 hours. Because P-Ser46 p53 is known to selectively induce mitochondrial p53AIP1 protein, we examined its level in MCF-7 cells exposed to roscovitine for increasing periods of time. p53AIP1 protein appeared in MCF-7 cells after 6 hours and remained up-regulated for at least 10 hours. A higher P-Ser46 p53 level correlated with the stronger up-regulation of p53AIP1 protein. Interestingly, monitoring of the intracellular distribution of the mitochondrial p53 targets revealed that newly synthesized p53AIP1 protein first appeared in the cytosol at 6 hours and then translocated into the mitochondria. The transcriptional induction of p53AIP1 protein that temporally preceded the release of distinct mitochondrial proteins into cytosol implicates its direct involvement in the dissipation of mitochondrial membrane. Released cytochrome c could bind to APAF-1 protein and complex to apoptosome. The apoptosome formation could be facilitated by an increase of APAF-1 concentrations in cytosol upon roscovitine. Moreover, the accumulation of apoptogenic proteins such as AIF (42), Smac/Diablo (43, 44), and mature HtrA2/Omi protease (45) in cytosol could additionally accelerate the execution of apoptosis. The central players in the apoptotic pathways are the caspases. Due to its destructive nature, caspase activity has to be tightly regulated. For activation of caspases, their processing is necessary but not sufficient. The endogenous inhibitors of caspases, representing the family of IAP characterized by the presence of baculovirus IAP repeat domains can bind and block active caspases. X-linked IAP, a member of this family, physically interacts through baculovirus IAP repeat domains with activated caspases-3, -7 and -9 and inhibits their activity (46). However, these complexes can be efficiently destabilized by Smac/Diablo (43, 44). HtrA2/Omi, a member of a novel family of mammalian serine proteases homologous to the bacterial HtrA gene product, seems to be a new player in this complex interaction network between caspases, inhibitors, and regulators. The HtrA2/Omi synthesized as a precursor has been reported as being localized either within the endoplasmic reticulum, the nucleus, or the mitochondrion (45, 47). After transport into mitochondria, the aminoterminal 133 amino acid fragment is cleaved off generating the mature protein (47). It has been shown that processed HtrA2/Omi translocated from mitochondria to the cytosol during apoptosis (47). The HtrA2/Omi release into the cytosol was restricted to cells dying by apoptosis. No release of mature HtrA2/Omi was detected in cells that die...
108 amino acids, respectively. Because p53AIP1 is involved in the interplay between p53 and MDM-2 protein, the suppressor p53 is inducible by Ser46-phosphorylated p53. The p53AIP1 correlates with the kinetics of depolarization of mitochondria. The phosphorylation of p53 at Ser46 in the former and caspase-3, is consistent with the roscovitine-induced apoptosis in both mRNA and protein levels (18). However, our observations are not fully consistent with the latter report. First, we found a strong enhancement of MDM-2 protein in MCF7 cells after roscovitine treatment. Furthermore, if the roscovitine-mediated suppression of MDM-2 would be a general phenomenon, one should expect a similar action of roscovitine on p53 in both MCF-7 cell lines irrespective of the caspase-3 status. However, we cannot exclude that other factors such as p14ARF protein are additionally involved in the interplay between p53 and MDM-2 protein after treatment with cyclin-dependent kinase inhibitors.

The transcriptional activation of p53AIP1 gene in parental MCF-7 cells, but not in cells reconstituted with caspase-3, is consistent with the roscovitine-phosphorylation of p53 at Ser46 in the former and correlates with the kinetics of depolarization of mitochondria. The p53AIP1 gene, a novel target for the tumor suppressor p53 is inducible by Ser46-phosphorylated p53 (24). The p53AIP1 gene generates three transcripts (α, β, and γ) by alternative splicing encoding peptides of 124, 86, and 108 amino acids, respectively. Because p53AIP1α and p53AIP1β are localized in mitochondria, they are potential mediators of mitochondrial membrane potential. Indeed, it has been shown that ectopic overexpression of p53AIP1 (24) induced apoptosis and adenovirus-mediated p53AIP1 gene transfer enhanced elimination of p53-resistant tumor cells by an increased apoptosis rate (48).

Our results strongly support the assumption that roscovitine induced activation and stabilization of p53 protein plays a key role in the initiation and regulation of apoptosis in MCF-7 cells. It seems that p53 controls not only dissipation of the mitochondrial potential by up-regulation of p53AIP1 protein but might also repress bcl-2 and reduce the apoptotic threshold by transactivation of caspases. There is an increasing body of evidence that initiator caspase-9 and executioner caspase-6 (49) are transcriptional targets of p53. Moreover, the activated p53 could enhance the expression of APAF-1 (50). Thus, the accumulation of APAF-1 in cytosol after roscovitine treatment might be a combinatory consequence of its transcriptional up-regulation as well as release from the mitochondria.

Taken together, activated p53 protein might positively affect the apoptosis in MCF-7 cells by two independent ways: by transcriptional modulation of the activity of several proapoptotic and antiapoptotic genes and by p53AIP1-mediated release of mitochondrial proteins involved in the complex interplay between caspases, inhibitors, and regulatory proteins. Because the latter pathway seems to be selectively induced in MCF-7 cells by site-specific phosphorylation of p53, it explains, at least partially, the reduced apoptosis rate in caspase-3-reconstituted cells. The more recent observation that specific down-regulation of X-linked IAP enhanced the efficacy of chemotherapeutic agents in MCF-7 cancer cells supports this assumption (51).

Our findings indicate that the tumor suppressor protein p53 controlling the cell division machinery and cell survival functions regulates very specifically the cellular response to the action of chemotherapeutic agents in individual cells. This assumption is in concordance with a more recent microarray study evidencing in breast cancer cell type-specific responses to chemotherapeutics (52).

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References


