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Journal of Cellular Biochemistry 105:1161–1171 (2008)

Journal of Cellular Biochemistry

Roscovitine Up-Regulates p53 Protein and Induces Apoptosis in Human HeLaS$_3$ Cervix Carcinoma Cells

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ABSTRACT

Exposure of human HeLaS$_3$ cervix carcinoma cells to high doses of conventional cytostatic drugs, e.g. cisplatin (CP) strongly inhibits their proliferation. However, most cytostatic agents are genotoxic and may generate a secondary malignancy. Therefore, therapeutic strategy using alternative, not cytotoxic drugs would be beneficial. Inhibition of cyclin-dependent kinases (CDKs) by pharmacological inhibitors became recently a promising therapeutic option. Roscovitine (ROSC), a selective CDK inhibitor, efficiently targets human malignant cells. ROSC induces cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. ROSC also activates p53 protein. Activation of p53 tumor suppressor protein is essential for induction of apoptosis in MCF-7 cells. Considering the fact that in HeLaS$_3$ cells wt p53 is inactivated by the action of HPV-encoded E6 oncoprotein, we addressed the question whether ROSC would be able to reactivate p53 protein in them. Their exposure to ROSC for 24 h induced cell cycle arrest at G$_2$/M and reduced the number of viable cells. Unlike CP, ROSC in the used doses did not induce DNA damage and was not directly cytotoxic. Despite lack of detectable DNA lesions, ROSC activated wt p53 protein. The increase of p53 levels was attributable to the ROSC-mediated protein stabilization. Further analyses revealed that ROSC induced site-specific phosphorylation of p53 protein at Ser46. After longer exposure, ROSC induced apoptosis in HeLaS$_3$ cells. These results indicate that therapy of HeLaS$_3$ cells by ROSC could offer an advantage over that by CP due to its increased selectivity and markedly reduced risk of generation of a secondary cancer. J. Cell. Biochem. 105: 1161–1171, 2008.

KEY WORDS: P53 TUMOR SUPPRESSOR; CELL CYCLE ARREST; CYCLIN-DEPENDENT KINASES; CYCLIN-DEPENDENT KINASE INHIBITORS; PHARMACOLOGICAL CDK INHIBITORS; E6 ONCOPROTEIN

Cisplatin [*cis*-diamminedichloroplatinum(II)] (CP) is a widely used drug for the treatment of various types of human malignancies. CP inhibits proliferation of human HeLaS$_3$ cervix carcinoma cells in a dose- and time-dependent manner [Fichtinger-Schepman et al., 1985; Cohen and Lippard, 2001] through cell cycle arrest in G$_1$ phase and via the induction of apoptosis by two independent ways: membrane death receptor-mediated and mitochondria-mediated pathways [Horky et al., 2000; Koivusalo et al., 2002; Wesierska-Gadek et al., 2002]. The anti-proliferative and pro-apoptotic action of CP become evident at relatively high concentrations. However, CP is strongly cytotoxic at higher concentrations and therefore, a treatment with alternative, more selective drugs that are low or not cytotoxic would strongly minimize side effects, thereby benefiting chemotherapy patients.

Progression of cells from one phase to the next occurs in an orchestrated fashion and is regulated by a number of cellular proteins. Cyclin-dependent kinases (CDKs) and cyclins, their specific regulatory proteins, play a key role [for review, see Wesierska-Gadek and Schmid, 2006]. Escape of cancer cells from the proper cell cycle control associated with the increased activity of growth-promoting factors allow them to rapidly proliferate. Therefore, different strategies targeting the major players of the cell cycle were

Abbreviations used: CDKs, cyclin-dependent kinases; CHX, cycloheximide; CP, cisplatin; DAPI, 4,6-diamidino-2-phenylindole; LMB, leptomycin B; MCM7, minichromosome maintenance 7; PARP-1, poly(ADP-ribose) polymerase-1; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; ROSC, roscovitine; WCL, whole cell lysate; WT, wild-type.

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Received 4 May 2008; Accepted 31 July 2008 • DOI 10.1002/jcb.21903 • 2008 Wiley-Liss, Inc.
Published online 7 October 2008 in Wiley InterScience (www.interscience.wiley.com).

developed. Therapeutic strategies include, among others, reactivation or overexpression of cellular CDK inhibitors and of p53 tumor suppressor, development of peptides mimicking the action of cellular CDK inhibitors, modulation of the proteasomal cascade, and manipulation of the activity status of cyclin–CDK complexes.

The direct inhibition of the catalytic components of the complexes seems to be a very efficacious strategy. Recently developed pharmacological inhibitors of CDKs are selective and act by competitive inhibition of ATP binding to CDKs. To date, a number of chemically and structurally diverging inhibitors with increased selectivity towards CDK1, CDK2, CDK4, and CDK5 are available. Purine analogues encompassing olomoucine and roscovitine (ROSC) represent a well-characterized group of CDK inhibitors [for review, see Wesierska-Gadek and Schmid, 2006]. ROSC, affecting primarily the activity of two kinases, namely CDK2 and CDK1, has been shown to efficiently target human malignant cells. ROSC arrests asynchronously growing human MCF-7 breast cancer cells at the transition between G_2 and M-phases [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Moreover, ROSC induces apoptosis in MCF-7 cells that are known to be relatively resistant to pro-apoptotic drugs due to the lack of caspase-3 activity [Jänicke et al., 1998]. ROSC activates wt p53 in MCF-7 cells and this seems to be an essential event for induction of apoptosis. Unexpectedly, ROSC activated HIPK2 [Wesierska-Gadek et al., 2007a] what induced the phosphorylation of p53 at Ser46 [Wesierska-Gadek et al., 2005a, 2007a]. The site-specific phosphorylation of p53 was crucial for transcriptional activation of the mitochondrial p53AIP1 protein that after de novo synthesis was involved in the execution of apoptosis [Wesierska-Gadek et al., 2005a].

Unlike in human MCF-7 breast cancer cells, in human $HeLaS_3$ cervix carcinoma cells wt p53 as well as the restriction checkpoint between G_1 and S phases are inactivated by the action of HPV-encoded E6 and E7 oncoproteins, respectively [Schwarz et al., 1985; Scheffner et al., 1990, 1993; Kessis et al., 1993]. Considering this fact and also a high efficacy of pharmacological inhibitors of CDKs in the therapy of different types of human cancer cells, we addressed the question whether ROSC would be able to negatively affect cell cycle progression in $HeLaS_3$ cells and to eliminate them by apoptosis. Furthermore, we raised the issue whether ROSC that does not induce DNA damage would be able to (re)activate p53 protein in HPV-positive cells despite the presence of E6 oncoprotein. We show in the present study that exposure of $HeLaS_3$ cells to ROSC for 24 h very efficiently reduces the number of viable cells and negatively affects their cell cycle progression. Remarkably, treatment of $HeLaS_3$ cells with ROSC results in an increase of the G_2 cell population and accumulation of hypoploid cells. Despite lack of detectable DNA lesions, ROSC activates wt p53 protein that, via upregulated $p21^{waf1}$, its downstream target, additionally enhances the ROSC-mediated inhibition of CDK2. After longer exposure, ROSC induces apoptosis in $HeLaS_3$ cells. Comparison of the p53 activation after administration of CP or ROSC revealed that both drugs stimulate phosphorylation of serine residues at different positions within the p53 molecule. ROSC induced primarily phosphorylation at Ser46, whereas CP induced it at Ser15. The above results indicate that the pharmacological inhibitor of CDKs is able to inhibit proliferation of $HeLaS_3$ cells by simultaneously affecting the cell cycle machinery, induction of apoptosis and reactivation of wt p53 which being at the crossroad of both pathways, may significantly contribute to their activation.

MATERIALS AND METHODS

CELLS

The human cervical carcinoma cell line $HeLaS_3$, obtained from American Type Culture Collection (ATCC), was cultured in RPMI medium supplemented with 10% foetal calf serum. Cells were grown up to 60–70% confluence and then treated with allocated drugs.

DRUGS

We used cisplatin (Lachema, Czech Republic) and ROSC, a selective CDK inhibitor. A 50 mM stock of ROSC was prepared in DMSO. Aliquots of stock solution were protected from light and stored until use at $-20^{\circ}C$.

CELL TREATMENT

Exponentially growing cells were exposed to CP or to ROSC at a final concentration ranging from 0.1 to 40 M for indicated periods of time.

ANTIBODIES

Monoclonal anti-PCNA (clone PC-10) antibodies were purchased from Oncogene Research Products (Cambridge, MA). Monoclonal anti-MCM-7 (DSC-141) and anti-B23 (C-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-p53 antibodies (DO-1) were from DAKO A/S (Glostrup, Denmark) and anti-actin antibodies were from ICN (ICN Biomedicals, Aurora, OH). Polyclonal anti-Ser46 p53 and anti-Ser15 p53 antibodies were from New England Biolabs (Beverly, MA). Fluorescein-conjugated mouse monoclonal antibodies M30 CytoDEATH recognizing only a caspase-3 cleaved cytokeratin 18 were from Roche Molecular Biochemicals (Vienna, Austria). Compatible secondary antibodies were obtained from Amersham International (Little Chalfont, Buckinghamshire, England).

DETERMINATION OF THE NUMBER OF VIABLE CELLS

The sensitivity of exponentially growing $HeLaS_3$ cells to increasing concentrations of CP or of ROSC was determined with the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). The CellTiter-Glo™ Luminescent Cell Viability Assay, generating luminescent signals, is based on quantification of the cellular ATP levels [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2004, 2005b]. Cells (5×10^3 cells/well) were plated into 96-well microtiter plates (two plates for each condition). One day after plating, cells were exposed to drugs for 24 h. Thereafter, the number of viable cells was determined in the first microtiter plate. In the second microtiter plate medium was changed and cells were post-incubated for a further 48 h in a drug-free medium. The measurement of the number of viable cells immediately after treatment for 24 h provided information on the direct cytotoxic effect of the drug. On the other hand, post-incubation of cells treated for 24 h, for another 48 h in a drug-free medium, allowed the evaluation of the long-term effects of the treatment. Tests were

performed at least in quadruplicate. Luminescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter. Each point represents the mean ± SEM (bars) of replicates from four experiments. Statistical analysis was performed using GraphPad Prism and significance levels were evaluated using *t*-test. IC_{50} values were evaluated from dose–response curve.

DETERMINATION OF THE VIABILITY OF CELLS

To discriminate between initial cell killing and inhibition of cell proliferation, the direct cytotoxicity of studied drugs was assessed by dye exclusion test [Wesierska-Gadek et al., 2004]. Cells were continuously treated with the studied compounds at indicated concentrations for 12 and 24 h. At the end of drug treatment, cellular morphology and the adherence of cells were evaluated under phase contrast microscopy (Eclipse TE300 inverted microscope, Nikon Corporation, Tokyo) and then cells were collected. The adherent cells were detached by trypsin or by accutase treatment (PAA Laboratories, GmbH, Coelbe, Germany) and all cells were washed with PBS. The vital dyes propidium iodide (PI) or 7-amino-actinomycin D (7-AAD) (BD Biosciences, San Diego, CA) appropriately diluted with PBS were added and after 10 min the accumulation of dyes was evaluated under fluorescence microscopy. Additionally, an accumulation of the fluorescent dyes was quantified by flow cytometry using a fluorescence activated cell sorter FACScan cytometer (Becton Dickinson).

DETECTION OF APOPTOTIC CELLS BY CytoDEATH STAINING

Apoptotic cells were detected by M30 CytoDEATH monoclonal antibody (Roche Molecular Biochemicals) recognizing caspase-3 cleaved cytokeratine 18. Apoptotic cells were detected in situ by indirect immunofluorescence microscopy and were additionally quantified by flow cytometric analysis [Wesierska-Gadek et al., 2005b]. For microscopic analysis cells were plated on slides in plastic chambers and appropriately cultivated. After treatment for indicated time, cells were washed three times in PBS, immediately fixed in ice-cold methanol, and stained. The fixed cells were incubated according to the manufacturer's protocol with the fluorescein-coupled monoclonal antibody M30. For visualization of nuclei, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) dissolved in PBS at a final concentration of 1 g/ml [Wesierska-Gadek et al., 2003, 2005b; Wojciechowski et al., 2003]. Cells were inspected under a fluorescence microscope (inverted microscope Eclipse TE300, Nikon Corporation, Tokyo).

MEASUREMENT OF THE DNA CONTENT OF SINGLE CELLS BY FLOW CYTOMETRY

The measurement of DNA content was performed by flow cytometric analysis based on a slightly modified method [Vindelov et al., 1983] from that described, previously [Wesierska-Gadek and Schmid, 2000]. The cells were detached from substratum by limited trypsinization, and then all cells were harvested by centrifugation and washed in PBS. Aliquots of 1×10^6 cells were used for further analysis. Cells were stained with propidium iodide as described, previously. Fluorescence was measured using the Becton Dickinson FACScan after at least 1 h incubation of the cells 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) as previously described [Kovar et al., 2000].

CELL FRACTIONATION

Nuclei were isolated from cells in logarithmic growth phase as previously described in detail [Wesierska-Gadek et al., 1995]. To avoid a proteolytic degradation during all isolation steps phenyl-methylsulfonylfluoride (PMSF) and Pefabloc were included at a final concentration of 1 mM and 50 M, respectively. Briefly, PBS-washed cells were suspended in ice-cold low salt buffer (RSB), swollen and after stepwise addition of detergents (NP-40 and freshly prepared sodium deoxycholate) were homogenized. After centrifugation through sucrose cushion purified nuclei were pelleted and solubilized in SDS-sample buffer.

DETERMINATION OF p53 STABILITY

Cells were grown in 100 mm Petri dishes for 24 h after plating to about 60–70% confluence. Cells were treated with 40 M ROSC for 15 h. Thereafter, controls, CP- and ROSC-treated cells were incubated with 10 M emetine or 20 g/ml cycloheximide (CHX) for various periods of time, washed with PBS and lysed in SDS sample buffer [Wesierska-Gadek et al., 1999, 2002, 2005b]. Equal amounts of whole cell lysates (WCLs) from each time point were separated on 10% SDS slab gels, immobilized on PVDF membrane, and subjected to immunoblotting using anti-p53 antibodies. The blot was then sequentially incubated with anti-PARP-1 antibodies. Equal protein loading was confirmed by Ponceau S staining of the proteins immobilized on the membrane and by sequential incubation of the membrane with anti-actin antibodies. The intensity of the p53 bands was determined by densitometric analysis of immunoblots and normalized against corresponding bands of actin.

IMMUNOBLOTTING

Proteins were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Immunodetection of antigens was performed with specific antibodies and enhanced chemiluminescent detection reagent ECL+ (Amersham International). Equal loading of proteins was confirmed by Ponceau S staining and additionally, by sequential incubation of blots with anti-actin antibodies [Wesierska-Gadek et al., 1995].

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism.

RESULTS

CP STRONGER INHIBITS THE PROLIFERATION OF HUMAN CERVICAL CANCER $HeLaS_3$ CELLS THAN ROSC

To compare the effect of both drugs on the number of living cells, exponentially growing $HeLaS_3$ cells were treated for 24 h with increasing concentrations of CP or ROSC. In agreement with previously published data [Horky et al., 2000; Wesierska-Gadek et al., 2002], treatment for 24 h with CP resulted in a clear reduction

of the number of viable cells in a time- and concentration-dependent manner (Fig. 1A). The number of viable cells was reduced by 50% at a final CP concentration of 3.71 M. ROSC displayed the same inhibitory effect at an approximately eightfold higher dose (28.2 M) (Fig. 1B). The IC_{50} difference assessed for both drugs was very highly significant. Moreover, to mimic the in vivo situation (treatment of patients), we exposed $HeLaS_3$ cells to both drugs at different concentrations for 24 h and then after medium change, cells were post-incubated in a drug-free medium for a further 48 h. The comparison of the action of CP directly after 24 h treatment and after 48 h post-incubation revealed a marked difference between short- and long-term effects of CP treatment (Fig. 1A). The IC_{50} value measured directly after 24 h treatment was 3.71 M CP and decreased nearly 6-fold after post-incubation for 48 h (0.6 M CP). The post-incubation of ROSC-treated $HeLaS_3$ cells for a further 48 h only slightly affected the number of viable cells. IC_{50} was reduced by approximately 30% (20.1 M) (Fig. 1B).

These results indicate that cells appearing to be viable after 24 h treatment with lower CP doses have been irreversibly damaged. Cells were unable to proliferate and died during further cultivation. Furthermore, the number of viable cells was also reduced during the post-incubation of ROSC-treated $HeLaS_3$ cells in a drug-free medium indicating that the inhibition of cell cycle progression persisted for a few days even in the absence of the drug. These results also indicate that the post-incubation time window offers an excellent opportunity for treatment with non-genotoxic agents that inhibit signal transduction processes and/or cell cycle progression to enhance the initial effect of strong cytostatic agents.

EXPOSURE OF HUMAN ASYNCHRONOUSLY GROWING $HeLaS_3$ CERVIX CARCINOMA CELLS TO ROSC AND TO CP DIFFERENTLY INHIBITS CELL CYCLE PROGRESSION

Considering the fact that in $HeLaS_3$ cells the restriction checkpoint is abolished by E7 oncoprotein, we raised the question how the inhibition of CDKs will affect their cell cycle. For comparison, the effect of CP, a strong cytostatic drug was examined. Determination of the DNA concentration in single cells showed that CP increases the ratio of $HeLaS_3$ cells in G_1 phase (Fig. 2A and B) and at higher concentrations induces the accumulation of a sub-G_1 cell population representing cells undergoing apoptosis (Fig. 2B). Unlike CP, ROSC blocked the exponentially growing $HeLaS_3$ cells in the G_2/M phase of the cell cycle in a time- and concentration-dependent manner (Fig. 2C). After exposure to 20 M ROSC for 24 h approximately 40% of cells were arrested at the G_2/M (Fig. 2C). The comparison of the G_1/S (Fig. 2B) and G_2/S (Fig. 2C) ratios substantiates the observation that both drugs differently affected the cell cycle progression of asynchronous $HeLaS_3$ cells. To discriminate between the G_2 and M block, the phosphorylation status of CDK1 as well as its activity was determined. Upon treatment with ROSC, phosphorylation of the inhibitory sites of CDK1 was enhanced, evidencing that cells were blocked in G_2 phase (not shown).

ROSC INDUCES APOPTOSIS

Accumulation of hypoploid cells usually implicates that cells undergo apoptosis. In the next experiments we determined the changes in the potential of the mitochondrial membrane and in the status of cytokeratin 18 in $HeLaS_3$ upon treatment. The M30 CytoDEATH antibody is known to selectively recognize cytokeratin 18 cleaved by caspase-3 and is highly specific for cells undergoing apoptosis. As depicted in Figure 3A, exposure to CP and to ROSC disrupted the potential of the mitochondrial membrane resulting in the loss of the ability to form J-aggregates. Moreover, it became also evident that both drugs induce apoptosis. However, the rate and kinetics of the apoptotic process strongly depends on the applied drug (Fig. 3A). Twenty-four hours after onset of CP treatment in approximately 50% of $HeLaS_3$ cells an accumulation of monomeric dye (green fluorescence) was observed. After ROSC treatment for 24 h the frequency of cells accumulating the monomeric dye was

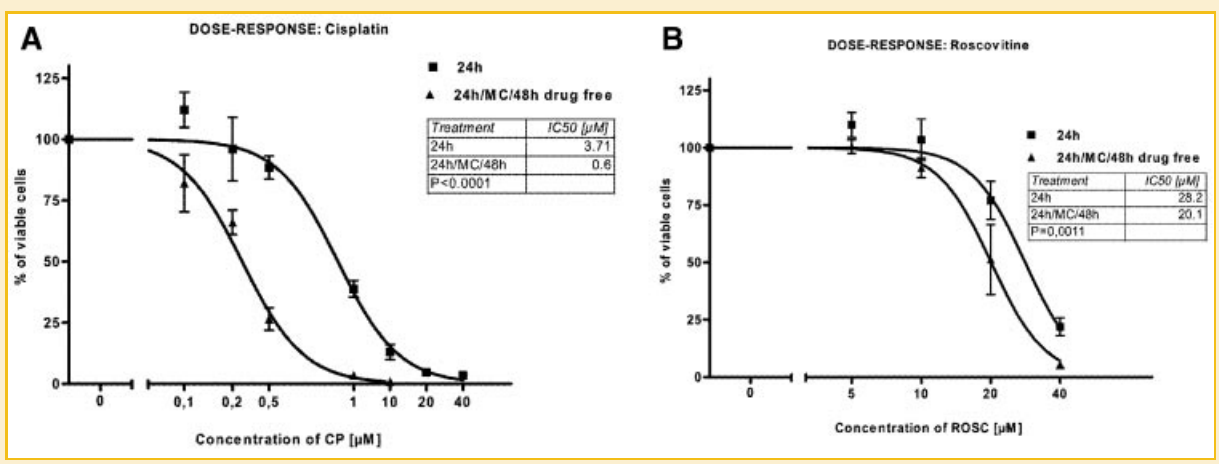

Fig. 1. CP stronger reduces the number of viable human $HeLaS_3$ cervical cancer cells than ROSC. Exponentially growing $HeLaS_3$ cells plated in 96-well microtiter plates were treated for 24 h with increasing concentrations of ROSC and CP. The number of viable cells was determined directly after treatment ((■) 24 h) and additionally, after post-incubation for 48 h in a drug-free medium ((▲) 24 h/MC/48 h). The graph represents mean values ± SEM from four independent experiments, each performed at least in quadruplicates. $IC_{50} = 3.71$ M CP after 24 h treatment; $IC_{50} = 0.6$ M CP after treatment for 24 h, medium change and post-incubation for 48 h. The difference of IC_{50} values is statistically highly significant ($P < 0.0001$).

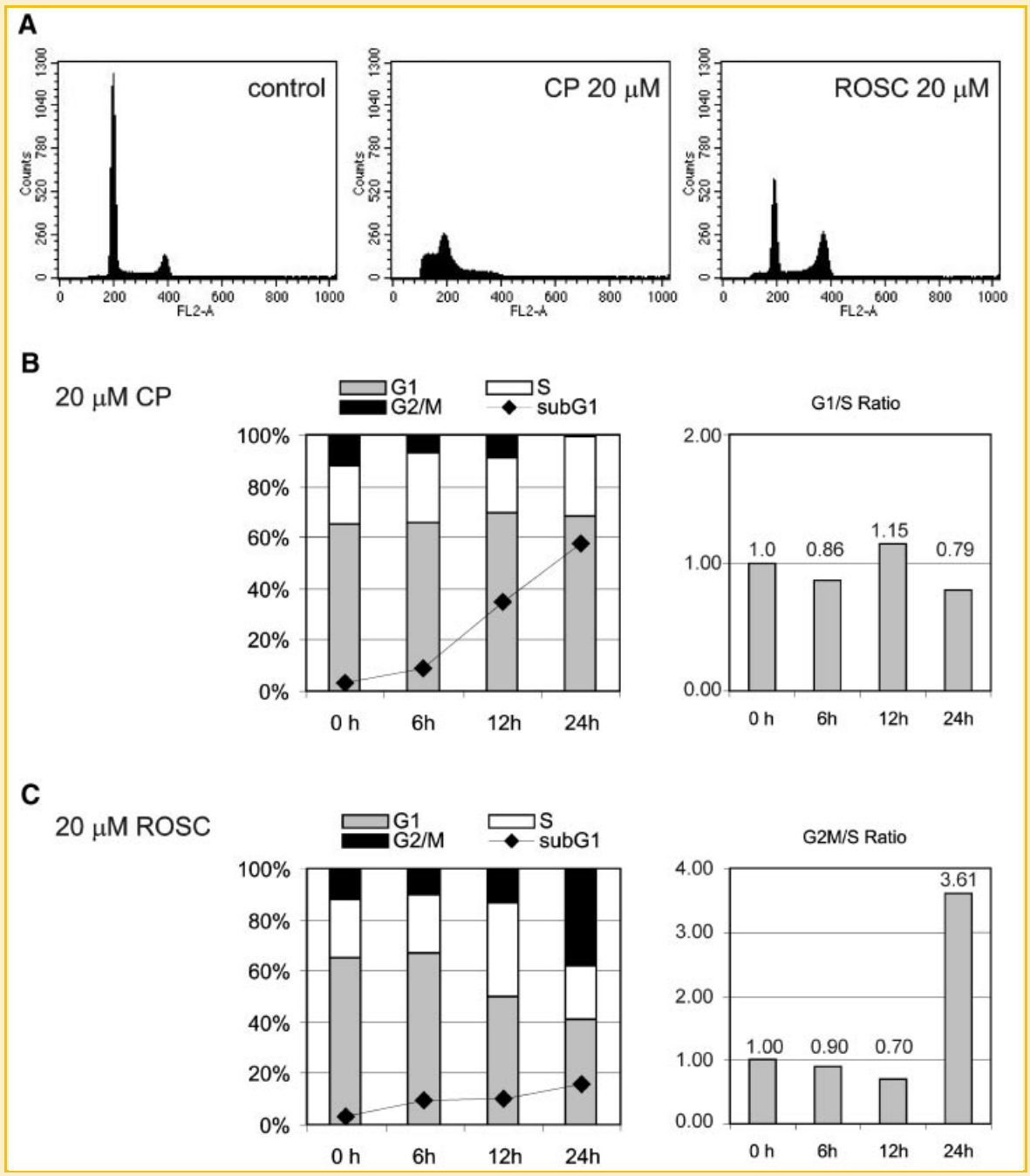

Fig. 2. Both CP and ROSC arrest the cell cycle in HeLaS$_3$ cells, but in different phases. The cells were treated with 20 M CP or 20 M ROSC for the indicated periods of time. A: Example FACS profiles of propidium iodide-stained control cells, and cells treated with CP or ROSC, respectively. B: CP at higher concentrations blocks HeLaS$_3$ cells in G$_1$ phase of the cell cycle and induces apoptosis, with G$_1$/S ratio almost unchanged. C: ROSC arrests HeLaS$_3$ cells in G$_2$/M phases of the cell cycle and strongly increases G$_2$/S ratio.

10-fold lower and increased after post-incubation in a drug free medium thereby evidencing that delayed effects occur after inhibition of CDK2.

Determination of number of cells stained by CytoDEATH representing cells in which cytokeratin 18, a constituent of cytoskeleton is cleaved by activated caspase-3, revealed that after treatment with 20 M CP for 24 h approximately 50% of cells were positively stained. After exposure for 24 h to 20 M ROSC the ratio of cells CytoDEATH stained cells was eightfold lower (Fig. 3B).

ROSC UP-REGULATES wt p53 PROTEIN IN HeLaS$_3$ CELLS

Although there is no mutation of the *p53* gene in human HeLaS$_3$ cells, its product is virally inactivated. Due to the activity of HPV-encoded E6 oncoprotein in HeLaS$_3$ cells the wt p53 protein is not functional. However, it has been previously reported that p53 can be

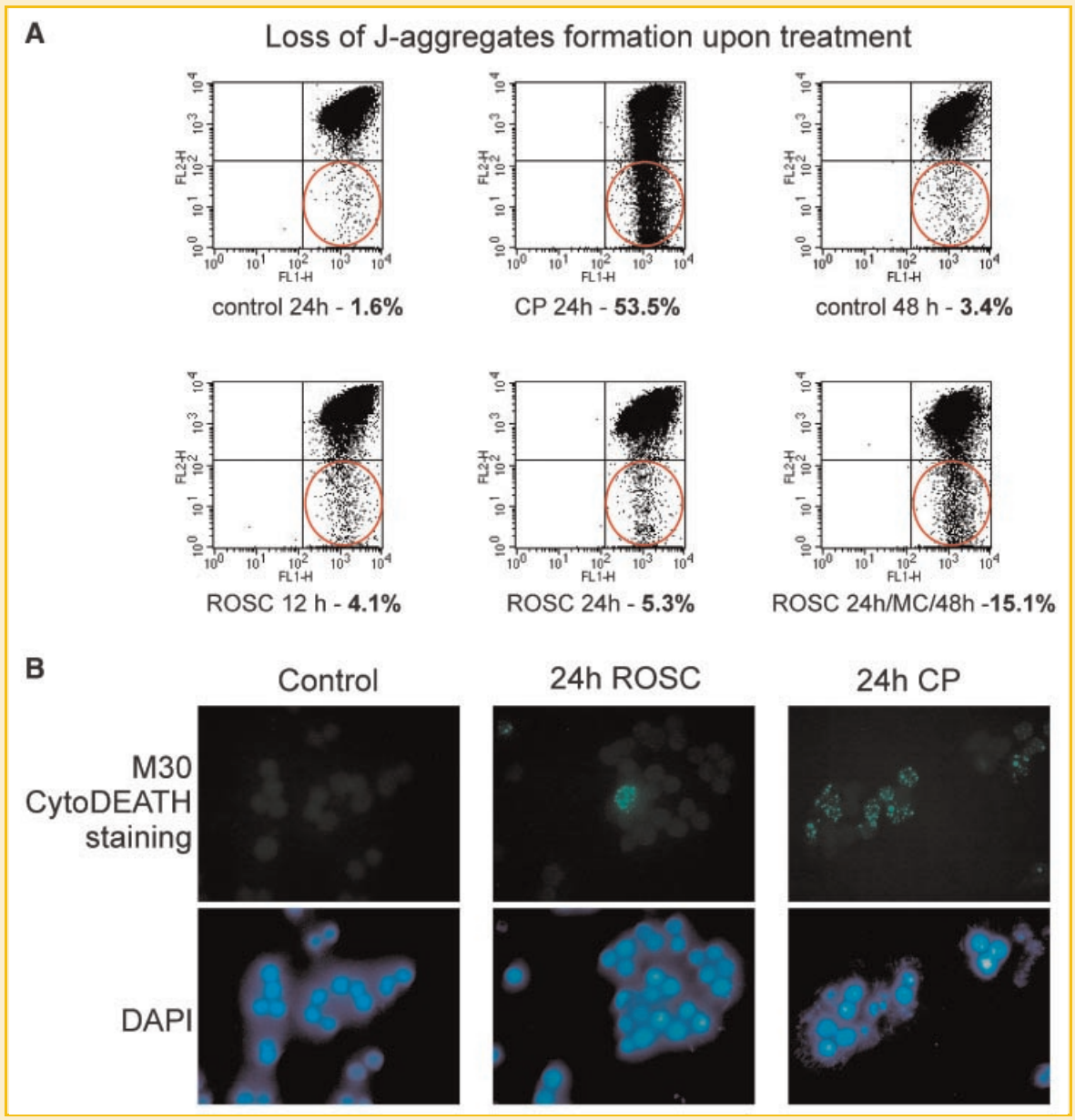

Fig. 3. Exposure of $HeLaS_3$ cells to CP and ROSC induces apoptosis. A: Bivariate JC-1 analysis of mitochondrial membrane potential in $HeLaS_3$ cells by flow cytometry. B: Detection of M30 CytoDEATH positive $HeLaS_3$ cells upon treatment with ROSC and CP. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

reactivated upon treatment with CP [Wesierska-Gadek et al., 2002, 2007; Schloffer et al., 2003]. Therefore, we examined the effect of ROSC treatment on the cellular expression of p53 protein in $HeLaS_3$ cells. As shown in Figure 4A, exposure of $HeLaS_3$ cells to 20 M ROSC for 15 and 24 h up-regulated cellular levels of p53 protein and resulted in its nuclear accumulation. This effect seems to be specific, because inhibition of nuclear export by leptomycin B (LMB) did not affect p53 concentration (Fig. 4A). In the next experimental series the time-course of p53 accumulation after exposure to ROSC was examined (Fig. 4B). Interestingly, the level of p53 protein increased very slowly and after 6 h only a very weak p53 signal was detected in the isolated nuclei but not in whole cell lysates (Fig. 4A). Unlike in ROSC-treated $HeLaS_3$ cells, in cells exposed to CP the accumulation of cellular p53 protein was observed already after 3 h (Fig. 4C).

The further analyses revealed that the up-regulated p53 protein was active as a transcription factor. It increased the expression of its downstream target such as $p21^{waf1}$ (not shown). Thus, these results indicate that exposure of $HeLaS_3$ cells to ROSC (re)activates cellular p53 pathways and in this way may additionally affect the progression of the cell cycle.

ROSC STABILIZES wt p53 PROTEIN IN $HeLaS_3$ CELLS

The tumor suppressor protein p53 is a short-lived protein and in $HeLaS_3$ cells its steady-state is markedly reduced by E6 oncoprotein.

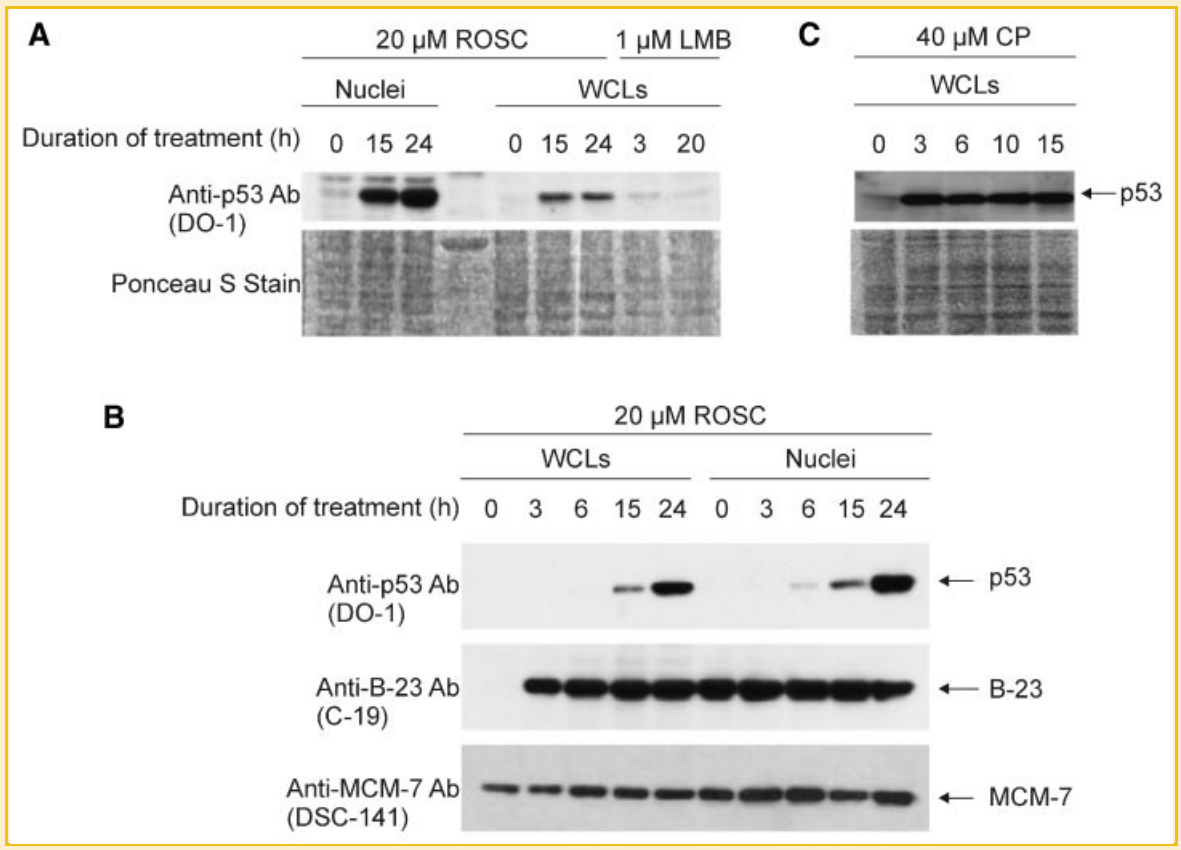

Fig. 4. Unlike CP, ROSC slowly increases cellular levels of p53 protein in human HeLaS_3 cells. A and B: ROSC up-regulates cellular levels of p53 protein, which is localized to the nucleus. C: Accumulation of p53 upon CP treatment occurs already within 3 h.

To assess the effect of ROSC on the p53 half-life, untreated cells and cells exposed to ROSC for 15 h were treated with emetine to block protein synthesis and then post-incubated for different time periods. Then WCLs were prepared and analyzed by immunoblotting. As depicted in Figure 5A, the p53 signal was very weak in untreated controls and it completely disappeared after a chase for 20 min. (Fig. 5A). However, in HeLaS_3 cells exposed to ROSC for 15 h, a strong p53 signal detected immediately upon addition of emetine (0′) did not decrease within a short time (Fig. 5A) and was reduced by 40% after post-incubation for 90 min. as assessed by densitometric evaluation (not shown). This clearly evidences that ROSC stabilized p53 protein in HeLaS_3 cells. CP increased the stability of p53 already after treatment for 5 h (Fig. 5B).

ROSC INDUCES SITE-SPECIFIC PHOSPHORYLATION OF p53 IN HeLaS_3 CELLS

The increase of p53 protein in ROSC-treated HeLaS_3 cells implicates that ROSC rendered p53 less susceptible to the E6–E6AP mediated polyubiquitylation and sequential degradation. The determination of the modification status revealed that ROSC induced phosphorylation of p53 protein at Ser46, whereas CP increased primarily phosphorylation of Ser15 (Fig. 5C). The different pattern of drug-induced p53 modification seems to be related to the stimulus-dependent activation of distinct cellular kinases.

CP BUT NOT ROSC INDUCES SITE-SPECIFIC PHOSPHORYLATION OF γ-H2AX PROTEIN

Finally, we determined the phosphorylation of -H2AX protein. It is widely established that after generation of DNA strand breaks, -H2AX protein becomes phosphorylated at Ser139. In untreated controls, the phosphorylation of H2AX protein is hardly detectable. After CP treatment, even at low concentrations, the phosphorylation of -H2AX protein at Ser139 strongly increases (not shown). On the other hand ROSC does not induce the site-specific phosphorylation of -H2AX protein thereby evidencing that contrary to CP, it does not generate DNA strand breaks.

DISCUSSION

CP is one of the most commonly used chemotherapeutic drugs, especially for the treatment of cervical carcinoma [for review, see Cohen and Lippard, 2001]. CP binds to DNA, primarily to guanine residues, resulting in formation of adducts and in cross-linking DNA to proteins [Fichtinger-Schepman et al., 1985]. CP-generated adducts lead to a change of DNA conformation and by this way attract nuclear proteins, most frequently those possessing a high mobility group domain [Huang et al., 1994]. It has been reported that CP-modified DNA is poorly repaired probably due to a shielding

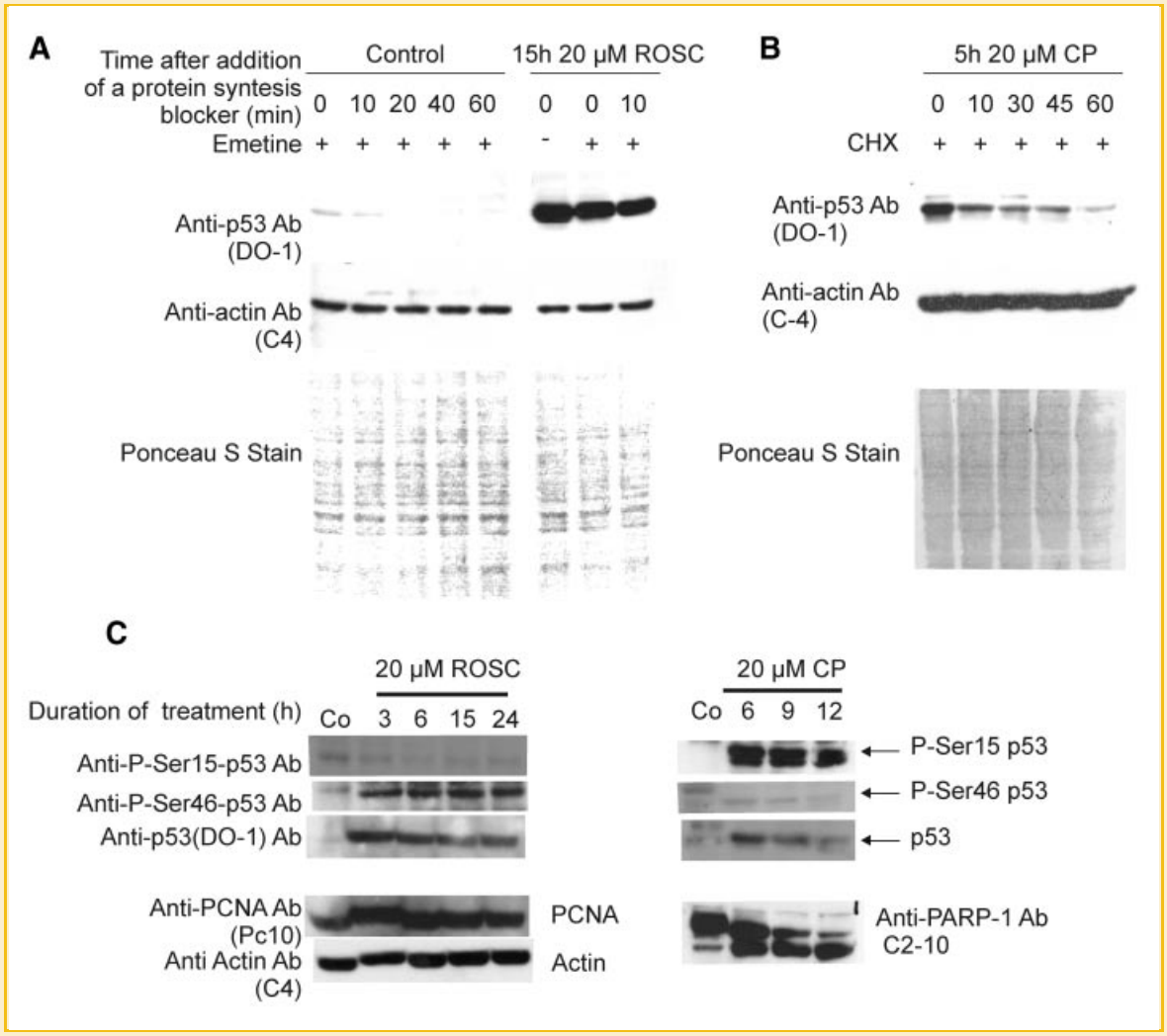

Fig. 5. ROSC stabilizes p53 and induces site-specific phosphorylation of p53 protein in $HeLaS_3$ cells. Control cells and cells exposed for 15 h to ROSC (A and C) and for 5 h to CP (B) were treated with inhibitors of protein synthesis (emetine or CHX) (A and B). WCLs were prepared at different time points after addition of the protein synthesis blockers. Proteins were resolved on 10% SDS gels and transferred onto PVDF membrane. Blots were incubated with antibodies as indicated. Proper transfer of proteins was checked by Ponceau S staining and equal protein loading was confirmed by incubation with anti-actin antibodies. Phosphorylation of p53 at Ser15 and Ser46 was determined using polyclonal phospho-specific antibodies. Sequential incubation with monoclonal anti-p53 antibodies visualized the total amount of p53 protein irrespective of post-translational modifications.

effect caused by these proteins [Fichtinger-Schepman et al., 1988; Zamble et al., 1996]. Chemotherapeutic action of CP relies primarily on its ability to induce apoptosis in tumor cells [Sorenson et al., 1990; Ormerod et al., 1994a,b; Horky et al., 2000; Schloffer et al., 2003; Yim et al., 2006]. Apoptosis triggered by CP is generally considered to be generated by block of replication and transcription as well as formation of reactive oxygen intermediates [Sorenson and Eastman, 1988]. However, the CP-mediated inhibition of replication is not a prerequisite for the initiation of apoptosis. We have previously observed that after exposure of $HeLaS_3$ cells to CP for 3 h apoptosis was induced and at this time a substantial number of mitotic cells was observed [Schloffer et al., 2003] indicating that in a short window of time both processes occurred simultaneously. The CP-triggered apoptosis was executed in two phases. In the early stage beginning in single cells at 3 h post-treatment an accumulation of cytochrome C in the cytosol and recruitment of Apaf-1 led to activation of caspase-9 and formation of the apoptosome [Horky et al., 2000; Schloffer et al., 2003]. Moreover, in CP-treated cells, p53 tumor suppressor was reactivated [Wesierska-Gadek et al., 2002]. However, the concentrations of CP that are required to induce a marked apoptotic response and to reactivate p53 protein in $HeLaS_3$ cancer cells are relatively high and therefore, a concomitant damage of healthy cells represents an undesired side effect of the CP chemotherapy. CP's cytotoxicity is dose-dependent and may occur within hours to days after its administration [Buhrer et al., 1990]. Therefore, clinical usefulness of the drug is strongly limited by severe complications like ototoxicity, neurotoxicity and nephrotoxicity [reviewed in Rybak, 2007; Yao et al., 2007]. Several efforts have been made to identify the mechanism(s) of the tissue injury and to develop new approaches preventing the strong toxicity

of the drug. CP-induced organ toxicity is generally associated with generation of free radicals especially reactive oxygen species that impair proteins, DNA, and lipids and can induce apoptosis in target tissues [Sorenson and Eastman, 1988]. To develop a protective strategy, the effects of co-administration of numerous antioxidant agents such as glutathione esters and amifostine were tested in animal models [Ekborn et al., 2004; Minami et al., 2004]. Unfortunately, clinical trials performing amifostine and CP co-administration were disappointing [Ekborn et al., 2004].

In contrast, targeted therapeutic options are usually more selective and are most frequently associated with no or strongly reduced cytotoxicity. Inhibition of CDKs represents such a selective therapeutic strategy. ROSC has received recently much attention due to its pleiotropic actions [Wesierska-Gadek and Schmid, 2006]. It not only inhibits CDK2, but also prevents phosphorylation of RNA polymerase II which is necessary for its induction and activates wt p53 protein [Wesierska-Gadek et al., 2005b, 2007]. These actions of ROSC result in distinct outcomes: inhibition of cell proliferation due to cell cycle arrest and/or initiation of apoptosis.

To clarify whether inhibitors of CDKs would also be effective as a weapon against HPV-positive cervix carcinoma, we examined the effect of ROSC on human $HeLaS_3$ cells. Our results indicate that the action of ROSC on cell cycle progression seems to depend on cell type and their intrinsic features. In asynchronously growing $HeLaS_3$ cells ROSC induced an accumulation of the G_2/M cell population. Analysis of the activity and phosphorylation status of CDK1 revealed that accumulation of G_2/M cell population was associated with an increase in its inhibitory phosphorylation sites thereby evidencing that ROSC blocked cells in the G_2 phase. Unlike in MCF-7 cells [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2008], in $HeLaS_3$ cells the ROSC-mediated G_2 arrest progressed slowly and a substantial increase of the number of G_2 cells was observed after 24 h.

Interestingly, ROSC markedly enhanced the cellular level of p53 tumor suppressor protein. The effect of ROSC seems to be specific, because inhibition of nuclear export by leptomycin B (LMB) in the same experimental series had a much weaker effect. LMB, interfering with the complex formation between CRM1 and p53, prevents the nuclear export and the degradation of the latter [Stommel et al., 1999]. The observed action of ROSC on p53 in $HeLaS_3$ cells is surprising for two reasons. First, what could be a trigger to (re)activate p53 cellular response since purine-based CDK inhibitors such as ROSC do not induce DNA damage, and secondly, how ROSC was able to prevent destructive p53 targeting by HPV-encoded E6. The degradation of p53 tumor suppressor protein by E6 activated cellular E6AP is highly dominant in human $HeLaS_3$ cervix carcinoma cells and for this reason p53 tumor suppressor protein is generally difficult to induce and was not detectable even after severe DNA damage induced by MNNG [Wesierska-Gadek et al., 2003] or by some anti-cancer drugs [Wesierska-Gadek et al., 2008a]. Moreover, inhibition of proteasomal activity is not sufficient to increase p53 levels in $HeLaS_3$ cells. We have previously observed that only one out of four used proteasomal inhibitors was able to increase cellular p53 levels in $HeLaS_3$ cells [Wesierska-Gadek et al., 2002].

In the light of the above considerations it was important to address the question by which mechanism ROSC is able to increase the p53 tumor suppressor protein in $HeLaS_3$ cells. Detailed analyses revealed that ROSC markedly stabilizes p53 protein in $HeLaS_3$ cells and induces site-specific phosphorylation. Modification of two serine residues of p53 was determined: at the positions 15 and 46. CP strongly induced phosphorylation of p53 at Ser15 but not at Ser46, whereas ROSC displayed the opposite action. These results are in concordance with the data on phosphorylation of -H2AX reflecting the damage of DNA and implicate that ROSC and CP stabilized p53 in $HeLaS_3$ cells by distinct pathways.

Remarkably, it has been demonstrated that E6 does not induce ubiquitylation of p53 in the same way as HDM2 in order to promote its degradation, suggesting important differences between the HDM2 and E6 effects on p53 degradation [Camus et al., 2003]. Indeed, a very recent report provides the first evidence of an E6-dependent, ubiquitin-independent p53 degradation pathway in vivo [Camus et al., 2007].

We have previously reported that the escape of wt p53 from E6-E6 AP-mediated degradation after treatment of $HeLaS_3$ with cisplatin [Wesierska-Gadek et al., 2002] and other anti-cancer drugs [Wesierska-Gadek et al., 2008a] strongly depends on their capacity to block the transcription of virally encoded proteins. This is in concordance with the observation that repression of virally encoded E6 protein in HPV positive cervix carcinoma cells promoted induction of apoptosis [Butz et al., 1996; Wesierska-Gadek et al., 2002; Allen et al., 2007].

Interestingly, it has been reported that pharmacological CDK inhibitors, such as purine-based ROSC, prevent the initiation of transcription from viral genomes [Schang et al., 2005]. Our preliminary results seem to confirm this mechanism of action. Repression of E6 levels was observed after longer exposure of $HeLaS_3$ cells to ROSC [Wesierska-Gadek et al., 2008b]. In our ongoing experiments we address the link between anti-viral action of ROSC and its capacity to restore the G_1/S checkpoint.

Thus, unlike CP, ROSC treatment represents a selective therapeutic option by affecting primarily transformed but not normal, healthy cells. It has been previously evidenced that transient inhibition of CDK2 in normal human cells neither induced marked inhibition of cell proliferation nor initiated apoptosis [Wesierska-Gadek et al., 2007b]. Considering the fact that cancer cells not only rapidly divide but also most frequently do not undergo apoptosis due to perturbations of programmed cell death, such as inactivation of caspases and/or overexpression of the inhibitors of apoptosis (IAPs), the administration of ROSC offers the possibility to simultaneously affect these dysfunctional processes in cancer cells. By selective inhibition of CDK2 and transcriptional CDKs at lower doses of ROSC the cell cycle progression is inhibited and the high levels of anti-apoptotic proteins are reduced. Moreover, (re)activation of the p53 tumor suppressor protein additionally contributes to inhibition of the cell cycle as well as to initiation and execution of apoptosis via transcriptional up-regulation of $p21^{waf1}$ and inducers and effectors of apoptosis or repression of IAPs such as survivin [Wesierska-Gadek and Schmid, 2007]. Furthermore, in virally transformed cells, e.g. human $HeLaS_3$ cells, ROSC-mediated inhibition of the transcription from viral genome restores tumor suppressors and checkpoints and prevents cell division errors

attributable to the E7-induced centriole overduplication [Duensing et al., 2007].

ACKNOWLEDGMENTS

The authors thank Dr. I. Herbacek for performing flow cytometric measurements and Mr. P. Breit for preparation of a photomicrograph of the immunostaining. This work was supported at part by a grant P-19894-B11 from the FWF.

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