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# Reconstitution of Human MCF-7 Breast Cancer Cells With Caspase-3 Does Not Sensitize Them to Action of CDK Inhibitors

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## ABSTRACT

Human MCF-7 breast cancer cells are resistant to pro-apoptotic stimuli due to caspase-3 inactivation. On the other hand, they should be sensitive to agents like selective pharmacological inhibitors of cyclin-dependent kinases (CDKs) that (re)activate p53 tumor suppressor protein because they harbor intact p53 pathways. In this study we examined whether reconstitution of caspase-3 in MCF-7 cells sensitizes them to inhibitors of CDKs, by analyzing the effects of roscovitine (ROSC) and olomoucine (OLO), two closely related selective pharmacological CDK inhibitors, on both mother MCF-7 cells and a secondary mutant line, MCF-7.3.28 that stably expresses human caspase-3. The results show that ROSC is, as expected, much more potent than OLO. Surprisingly; however, ROSC and OLO reduced proliferation of parental MCF-7 cells more strongly than caspase-3-proficient counterparts. Both inhibitors arrest human breast cancer cells at the $G_2$-phase of the cell cycle. Analysis of cell-cycle regulators by immunoblotting revealed that ROSC strongly induces p53 protein activity by inducing its phosphorylation at Ser46 in the MCF-7 cells lacking caspase-3, but not in caspase-3-proficient cells. Furthermore, reconstitution of caspase-3 in MCF-7 cells neither elevates the mitochondrial apoptosis rate nor significantly increases caspases activity upon ROSC treatment. However, the stabilization of p53 in response to DNA damaging agents is the same in both caspase negative and positive MCF-7 cells. Cytotoxic agents induce caspase-3-dependent apoptosis in caspase-3-proficient cells. These results indicate that reconstitution of MCF-7 cancer cells with caspase-3 sensitize them to the action of DNA damaging agents but not to ATP-like pharmacological inhibitors of CDKs. J. Cell. Biochem. 112: 273–288, 2011. 

**KEY WORDS:** CELL-CYCLE ARREST; CDK INHIBITORS; G2 ARREST; P53AIP1

Human breast cancer is characterized by various perturbations in cellular signaling pathways [Sutherland and Musgrove, 2004]. Upregulation of cyclin D [Schuuring et al., 1992; Couse and Korach, 1999] and HER2 [Murphy and Fornier, 2010], as well as constitutive activation and/or overexpression of estrogen receptor-α are very frequent events that affect cell-cycle regulation, cell proliferation, and apoptosis in these cancers [Nathanson et al., 2001]. Inactivation of cellular inhibitors of cyclin-dependent kinases (CDKs) during malignant transformation also contributes to the escape of breast cancer cells from cell-cycle control [Sherr and Roberts, 1999; Blagosklonny and Pardee, 2001; Senderowicz, 2001; Negrini et al., 2010]. The defects in cell-cycle regulation can be compensated by applying pharmacological inhibitors of CDKs [Lapenna and Giordano, 2009; Sutherland and Musgrove, 2009]. Despite initial skepticism, arising from the conserved site of ATP-binding proteins, a number of selective

Abbreviations used: AIF, apoptosis inducing factor; CKI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; CP, cisplatin; CTD, carboxy-terminal domain; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; HMC, Hoffman modulation contrast; HRP, horseradish peroxidase; IAP, inhibitor of apoptosis protein; MDM-2, mouse double minute-2; OLO, olomoucine; p53AIP1, p53-apoptosis inducing protein 1; PARP-1, poly(ADP-ribose) polymerase-1; PCNA, proliferating cellular nuclear antigen; PD, Petri dish; PVDF, polyvinylidene difluoride; RNA pol II, RNA polymerase II; ROSC, roscovitine; STAU, staurosporine; WCL, whole cell lysate; wt, wild-type.Recipient of a DOC-fFORTE–fellowship of the Austrian Academy of Science.

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CDK inhibitors have been developed in the last two decades [Lapenna and Giordano, 2009; Węsierska-Gądek et al., 2009c; Galons et al., 2010; Rizzolio et al., 2010]. The reported inhibitors include natural compounds, their derivatives, and agents developed during structure–activity relationship studies. The majority of reported CDK inhibitors display pan-specific affinity for a few kinases or even family of CDKs. Roscovitine (ROSC), a tri-substituted purine derivative (Seliciclib™; CYC-202), inhibits primarily CDK2, 5, 7, and 9 [Vesely et al., 1994; De Azevedo et al., 1997; Havlicek et al., 1997; Meijer et al., 1997]. Its biological effects depend on the cell type, concentration used, and duration of treatment [Węsierska-Gądek et al., 2009a]. Due to the pleiotropic effects, ROSC is very efficient not only against rapidly dividing cancer cells but also against resting cells like chronic lymphocytic leukemia (CLL) cells [Alvi et al., 2005; MacCallum et al., 2005; Paprskarova et al., 2009; Rogalinska et al., 2010]. We reported several years ago that ROSC strongly affects the proliferation and cell-cycle progression of chemoresistant human MCF-7 breast cancer cells [Wojciechowski et al., 2003]. It arrested MCF-7 cells at the $G_2$/M phase of the cell cycle and concomitantly induced apoptosis. Upon ROSC treatment the level of wt p53 protein strongly increased and p53 accumulated in the nucleus. MCF-7 cells are known to be caspase-3-deficient [Janicke et al., 1998b], arousing interest in the pathway involved in ROSC-induced apoptosis. ROSC induced a marked depolarization of mitochondrial potential, beginning 6 h after the start of the treatment and release of the mitochondrial proteins cytochrome *c* and apoptosis inducing factor (AIF) into the cytosol. Interestingly, ROSC stimulated phosphorylation of wt p53 protein at Ser46 in a time-dependent manner [Wesierska-Gadek et al., 2005a]. Wt p53 tumor suppressor activated by site-specific phosphorylation induced, in turn, transcriptional upregulation of p53-apoptosis inducing protein 1 (p53AIP1), its downstream target (which is known to mediate the depolarization of mitochondrial potential) [Wesierska-Gadek et al., 2005a]. The onset of phosphorylation of p53 at Ser46 preceded the upregulation of p53AIP1 protein and the depolarization of mitochondrial potential [Wesierska-Gadek et al., 2005a]. Thus, ROSC-induced caspase-3-independent cell death in MCF-7 cells.

In the present work we assessed whether reconstitution of MCF-7 cells with caspase-3 would sensitize them to inhibitors of CDKs by switching from caspase-3-independent to caspase-3-dependent apoptosis. For this purpose, we determined the effects of ROSC and olomoucine (OLO), two closely related selective pharmacological CDK inhibitors, on the proliferation and cell cycle distribution of exponentially growing asynchronous parental MCF-7 cells and secondary MCF-7.3.28 mutant cells stably expressing human caspase-3 [Janicke et al., 1998b]. Both pharmacological inhibitors of CDKs block the proliferation of human breast cancer cells in a time- and concentration-dependent manner. As expected, ROSC is more potent than OLO and a much lower dose (four- to sevenfold) is sufficient to reduce the number of living MCF-7 cells by 50%.

Unexpectedly, both pharmacological inhibitors of CDKs decrease proliferation of parental MCF-7 cells more strongly than caspase-3-proficient counterparts. More efficient inhibition of the proliferation of MCF-7 cells seems to be attributable to rapid and more pronounced induction of $G_2$ arrest upon ROSC treatment in parental caspase-3-deficient MCF-7 cells than in the caspase-3-proficient cells. Analysis of cell-cycle regulators by immunoblotting revealed that ROSC strongly induces p53 protein by inducing its phosphorylation at Ser46 in the mother cell line, but not in caspase-3-proficient cells. Furthermore, reconstitution of caspase-3 in MCF-7 cancer cells neither elevates the apoptosis rate nor significantly increases caspases activity upon ROSC treatment. These results indicate that caspase-3-proficient MCF-7 cells do not switch to caspase-dependent apoptosis upon ROSC treatment. These and other observations are reported and discussed below.

## MATERIALS AND METHODS

### DRUGS

The purine-derived CDK inhibitors ROSC and OLO were obtained from Prof. M. Strnad (Palacky University, Olomouc, Czech Republic). Cisplatin (CP) was from Lachema (Czech Republik), 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), etoposide (VP16), and staurosporine (STAU) were from Sigma–Aldrich (St. Louis, MO).

### CELLS AND TREATMENT

Human breast carcinoma MCF-7 cells, secondary mutant MCF-7.3.28 cells reconstituted with caspase-3 under the control of the CMV promoter and MCF-7.0.3 cells transfected with empty vector were used [Janicke et al., 1998a,b]. MCF-7 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% $CO_2$. The secondary mutant caspase-3-proficient cell line and cells transfected with control vector were maintained as previously described [Janicke et al., 1998a,b]. Cells were grown to 60–70% confluence and then treated with ROSC and OLO at concentrations ranging from 1 to 40 μM for indicated periods of time indicated in Figures 1–10. ROSC, OLO, DRB, STAU, and VP16, were dissolved as a stock solution in DMSO and stored at −20°C until use.

### ANTIBODIES

The following specific primary antibodies were used to detect the relevant proteins: monoclonal anti-p53 antibody DO-1 (a kind gift from Dr. B. Vojtesek, Masaryk Memorial Cancer Institute, Czech Republic), the polyclonal anti-phospho-Thr14/Tyr15 CDK1, anti-phospho-Thr160 CDK2, anti-phospho-Ser780 pRb, and corresponding antibodies against the total antigen (all from New England Biolabs, Beverly, MA), polyclonal anti-phospho-Ser164/Thr170 CDK7 (BioLegend, San Diego, CA), anti-caspase-3 (DAKO AS, Glostrup, Denmark), monoclonal anti-CDK2 (Ab-4) antibodies (Lab Vision Co., Fremont, CA), polyclonal anti-phospho-Thr34 survivin, anti-pRb (IF-8), mouse monoclonal anti-PCNA (PC-10), anti-poly(ADP-ribose) polymerase-1 (PARP-1) (C2-10), anti-p53AIP1, and anti-MCM7 (DSC-141) antibody (all from Santa Cruz Biotechnology, CA); anti-p53AIP1 antibody (AnaSpec, Inc., San Jose, CA), anti-CDK7 (clone MO-1.1) (Sigma–Aldrich), anti-RNA polymerase II (RNA pol II) phosphorylated on Ser5 (clone H14), and anti-RNA pol II phosphorylated on Ser2 (clone H5) (all from Abcam pIc, Cambridge); anti-RNA pol II (clone ARNA-3), ACRIS Antibodies GmbH, (Herford), and anti-actin (clone C4, ICN Biochemicals, Aurora, OH). Appropriate secondary antibodies linked

to horseradish peroxidase (HRP) were obtained from R&D Systems (Minneapolis, MN).

### DETECTION OF APOPTOTIC CHANGES IN SITU IN SINGLE CELLS

For microscopic investigations and evaluation of nuclear morphology, cells were plated on slides in chambers and appropriately cultivated. After treatment for indicated periods of time indicated in Figure 3, cells were washed three times in PBS, fixed in 3.7% paraformaldehyde in cacodylate buffer and stained with Hoechst 33258 dissolved in PBS at a final concentration of 1.5 μg/ml [Wesierska-Gadek et al., 2004b]. Preparations were then washed four times with PBS and finally air-dried. Thereafter, specimens were inspected under an Eclipse TE300 inverted fluorescence microscope (Nikon Corporation, Tokyo).

### DETERMINATION OF THE NUMBERS OF LIVING CELLS

The numbers of viable human breast cancer cells following the treatments and their sensitivity to the tested drugs at various concentrations were determined by CellTiter-Glo™ assays (Promega Corporation, Madison, WI). As described recently in more detail [Wesierska-Gadek et al., 2005b], in the CellTiter-Glo™ luminescent cell viability assay luminescent signals, which are correlated with cellular ATP levels, are measured.

Tests were performed at least in quadruplicate, and the cells' luminescence was measured in a Wallac 1420 Victor multilabel, multitask plate counter (Wallac Oy, Turku, Finland). Each data point represents the mean ± SD (bars) of replicates from at least three independent experiments. Effect of the post-incubation on the $IC_{50}$ values was determined and defined as a reduction factor (reduction factor = $IC_{50}$ after 24 h/$IC_{50}$ after 24 h/MC/p.i. for 48 h).

### MEASUREMENT OF DNA IN SINGLE CELLS BY FLOW CYTOMETRY

Cells' DNA contents were measured by flow cytometric analysis based on the method of Vindelov et al. [1983], with slight previously described [Wesierska-Gadek and Schmid, 2000] modifications. Briefly, the adherent 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 stained with propidium iodide as previously described; then their fluorescence was measured using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) after at least 2 h incubation at +4°C in the dark. Their DNA concentration was evaluated using ModFIT LT™ cell-cycle analysis software (Verity Software House, Topsham, ME) and DNA histograms were generated using CellQuest™ software (Becton Dickinson).

### 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]. Control and drug-treated cells were harvested, washed and incubated with the dye at a final concentration of 10 μM for 5 min followed by extensive washings in PBS and immediate two-color analysis by flow cytometry. JC-1 is accumulated in mitochondria as red fluorescent aggregates (J-aggregates) (excitation/emission at 488–570 nm, respectively) in intact cells. Upon mitochondrial depolarization, it occurs as green fluorescent monomers (excitation/emission at 488–530 nm, respectively).

### DETERMINATION OF THE ACTIVITY OF CASPASES

The activity of caspase-3/7 was determined using the Caspase-3-GLO Assay (Promega Corporation), with a luminogenic caspase-3/7 substrate harboring the caspase-3/7 DEVD sequence as previously described [Wesierska-Gadek and Schmid, 2000; Wesierska-Gadek et al., 2004b]. An equal volume of the caspase-3-GLO reagent was added and the samples were incubated at 37°C for different periods of time to assess the best signal-to-background ratio. The luminescence was measured at 30 min intervals.

The activity of caspase-9 was determined using the Homogenous Caspase-9 GLO Assay (Promega Corporation), in which a luminogenic caspase-9 substrate harboring the LEDH sequence is incubated with test samples. Following cleavage by caspase-9, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the generation of light. The luminescence, which is directly proportional to the amount of activated caspase-9, was measured using a Wallac 1420 Victor multifunction plate reader. Each presented data point represents the mean ± SD (bars) of at least three replicates.

### SUBCELLULAR FRACTIONATION OF CELLS

In experiments designed to examine the release of proteins specifically released from mitochondria during the apoptosis process, cells were fractionated according to the procedure described by Fiskum in which PBS-washed cells were permeabilized in a buffer containing 250 mM sucrose and a low concentration of digitonin [Fiskum et al., 2000].

### ELECTROPHORETIC SEPARATION OF PROTEINS AND IMMUNBLOTTING

Total cellular proteins or proteins of distinct subcellular fractions dissolved in SDS sample buffer were separated on 10% or 15% SDS slab gels, transferred electrophoretically onto polyvinylidene difluoride (PVDF) membrane (Amersham International, Little Chalfont, Buckinghamshire, England) and immunoblotted as previously described [Wesierska-Gadek et al., 2000, 2002]. 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 3% BSA in Tris-saline-Tween-20 (TST) buffer [Wesierska-Gadek et al., 2004b]. In some cases, blots were used for sequential incubations. Immune complexes were detected after incubation with appropriate HRP-coupled secondary antibodies using ECL Plus™ Western Blotting Reagents from GE Healthcare. This system utilizes chemiluminescence technology for the detection of proteins. Chemiluminescence was detected after exposing the blots to film or by analysis using ChemiSmart5100 apparatus (PEQLAB, Biotechnologie GmbH, Erlangen, Germany).

### STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) and significance levels were evaluated using Bonferroni's multiple comparison test and Dunnett's

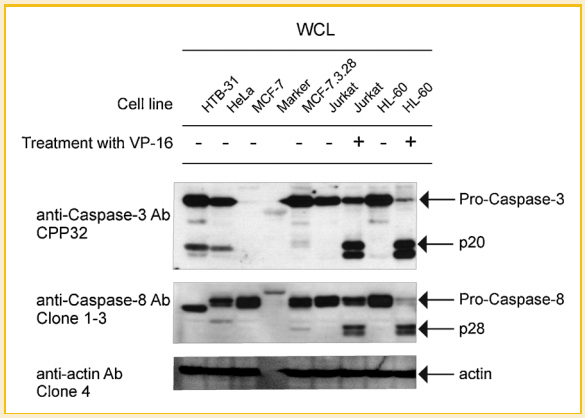


Fig. 1. After reconstitution caspase-3 is expressed in MCF-7.3.28 cells at a physiologic level. WCLs prepared from untreated human cancer cell lines and cells treated for 24 h with 10 μM etoposide (VP-16) were separated on 15% SDS gels and transferred onto polyvinylidene difluoride membranes (PVDF) (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England (formerly Amersham Biosciences)). The blots were incubated with indicated primary antibodies in an appropriate concentration. After incubation with secondary antibodies linked to HRP, immune complexes were detected by chemiluminescence using ECL Plus™ Western Blotting Reagents from GE Healthcare. This system utilizes chemiluminescence technology for the detection of proteins. Chemiluminescence was detected after exposing the blots to film or by analysis using ChemiSmart5100 apparatus (PEQLAB, Biotechnologie GmbH, Erlangen, Germany). Equal protein loading was confirmed by Ponceau S staining of the membrane and by incubation with anti-actin antibodies.

multicomparison test. Differences between treatments were deemed to be extremely significant, very significant, significant, and not significant if their $P$ values (according to Bonferroni's comparison test) were $<0.001$, $<0.01$, $0.01 < P < 0.05$, and $>0.05$, respectively.

## RESULTS

### AFTER RECONSTITUTION CASPASE-3 IS EXPRESSED IN HUMAN BREAST CANCER CELLS AT A PHYSIOLOGIC LEVEL

To assess the cellular level of caspase-3 after its reintroduction to human MCF-7 breast cancer cells, we analyzed by immunoblotting whole cell lysates (WCLs) prepared from several human cancer cell lines including parental cell line (MCF-7) and caspase-3 reconstituted cells (MCF-7.3.28). As shown in Figure 1, after reconstitution pro-caspase-3 was expressed in secondary MCF-7.3.28 cell line at a physiologic level comparable with that observed in other examined human cell lines. Moreover, the incubation of a twin blot with antibodies against caspase-8 confirmed comparable levels of this pro-zymogen in all tested cell lines. Furthermore, analysis of samples obtained from etoposide-treated leukemia cells (Jurkat and HL-60 cells) revealed that used anti-caspases antibodies recognize both pro-zymogens and their cleaved fragments (Fig. 1).

### ROSC INHIBITS PROLIFERATION OF CASPASE-DEFICIENT MCF-7 CANCER CELLS MORE STRONGLY THAN CELLS RECONSTITUTED WITH CASPASE-3

All three examined breast cancer cell lines divide very rapidly. Twenty-four hours after plating cells were exposed to a range of concentrations of OLO and ROSC. After treatment for 24 h, the numbers of living cells were determined immediately or, alternatively, after changing the medium and post-incubation of the cells in a drug-free-medium for 48 h. ROSC inhibited proliferation of the tested breast cancer cells more strongly than OLO (Table I); approximately fourfold less concentration of ROSC was sufficient to reduce the numbers of viable cells by 50% after 24 h (Table I). Interestingly, the anti-proliferative effects of the pharmacological CDK inhibitors, especially ROSC, were maintained after washout and post-incubation for 2 days (Fig. 2). There was a sevenfold difference in $IC_{50}$ values between ROSC and OLO after the post-incubation, indicating that ROSC-induced cell-cycle arrest is not transient, but is maintained for at least 2 days in the absence of the drug.

TABLE I. Comparison of $IC_{50}$ Values for MCF-7 Cell Clones

| | MCF-7 | MCF-7.0.3 | MCF-3.28 |
|---|---|---|---|
| Caspase-3 status | −/− | −/− | +/+ |
| ROSC | | | |
| 24 h | 39 | 36 | 39 |
| 24 h/MC/p.i. for 48 h | 16 | 20 | 25 |
| Reduction factor[a] | 2.4 | 1.8 | 1.5 |
| OLO | | | |
| 24 h | 141 | 150 | 157 |
| 24 h/MC/p.i. for 48 h | 108 | 114 | 122 |
| Reduction factor[a] | 1.31 | 1.32 | 1.29 |

MCF-7 cells were treated with CDK inhibitors for 24 h. The numbers of viable cells were determined immediately after treatment, or alternatively, after medium change (MC) and sequential post-incubation for 48 h in a drug-free medium. $IC_{50}$ values were established from dose–response curve generated using GraphPad Prism software (GraphPad Software, La Jolla, CA). Effect of the post-incubation on the $IC_{50}$ values was determined (Material and Methods Section) and defined as a reduction factor.
[a]Reduction factor = $IC_{50}$ after 24 h/$IC_{50}$ after 24 h/MC/p.i. for 48 h.

Furthermore, marked differences in the sensitivity of the tested breast cancer cell lines to the pharmacological CDK inhibitors became apparent. Our experiments revealed, unexpectedly, that after washout and post-incubation parental cells were more sensitive to CDK inhibitors than cells reconstituted with caspase-3 (Fig. 2). A higher concentration of ROSC was needed to reduce the numbers of viable caspase-3-proficient cells by 50% (Fig. 2 and Table I). The control cell line transfected with empty vector was also slightly less sensitive than parental cells, indicating that even transfection with empty vector slightly changes the sensitivity of the cells to the drugs. In addition, in soft agar tests (clonogenic assay) of the cytostatic effects of ROSC the drug significantly reduced colony numbers of all three cell lines (data not shown).

### A HIGHER ROSC DOSE IS NEEDED TO INDUCE $G_2$/M ARREST IN CASPASE-3-PROFICIENT BREAST CANCER CELLS

The measurements of DNA concentration in single cells revealed that ROSC blocks the examined breast cancer cells at the $G_2$/M transition in a concentration-dependent manner (Fig. 3). A final concentration of 20 μM was sufficient to block parental MCF-7 cells (42% cells at $G_2$); while at this dose ROSC increased the frequency of the $G_1$ population in MCF-7.3.28 cells by 13% (52% cells at $G_1$). At double concentration ROSC retarded the progression of parental MCF-7 cells from S- to $G_2$-phase (Fig. 3). These data are consistent with our previously published results [Wesierska-Gadek et al.,

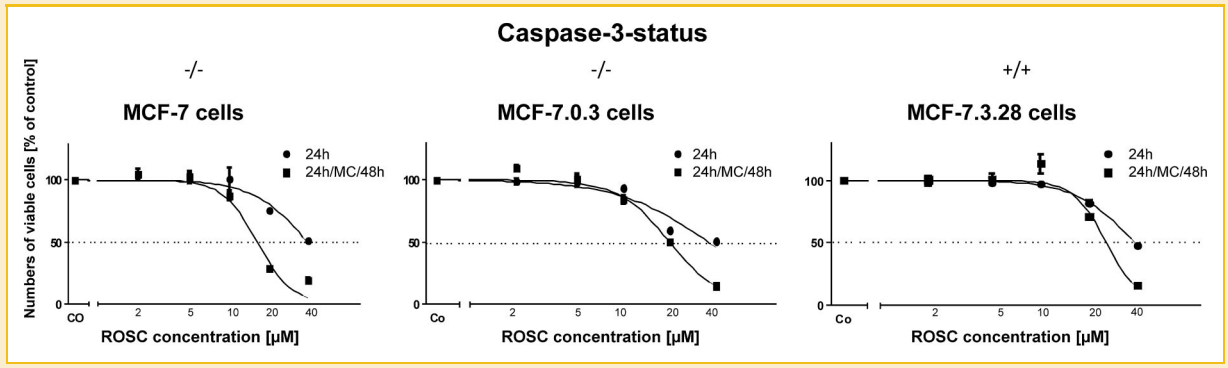


Fig. 2. Reduction of the numbers of viable human breast cancer cells after treatment with ROSC. Exponentially growing parental MCF-7 cells and two secondary lines (MCF-7.3.28 and MCF-7.0.3) plated in 96-well microtiter plates were treated for 24 h with indicated concentrations of ROSC. The numbers of viable cells were determined directly after the treatment, or after medium change (MC) and post-incubation for 48 h in a drug-free medium. These data represent mean values from three independent experiments, each performed at least in quadruplicate. The differences between the number of control and treated cells are statistically highly significant ($^{**}$; $0.001 < P < 0.01$) or very highly significant ($^{***}$; $P < 0.001$) according to the Bonferroni comparisons.

2005a]. The ROSC concentration had to be twice as high to block caspase-3-proficient cells at the $G_2$/M transition. The ROSC-induced changes in the cell-cycle progression were maintained in parental cells even after post-incubation in a drug-free medium for 24 and 48 h. Maintenance of this effect was more pronounced in cells released from treatment with 40 μM than 20 μM ROSC and seems to be attributable to the progression of cells accumulated in the S-phase into the $G_2$ in the absence of the CDK inhibitor. However, this long-term effect was much weaker in secondary mutant cells (Fig. 3). The numbers of MCF-7.3.28 cells arrested in $G_2$-phase upon treatment with 40 μM ROSC decreased after post-incubation in a drug-free medium for 1 or 2 days by approximately 15% (from 43% to 28%). Remarkably, the frequency of the population of S-phase cells increased after release from the drug. This is not surprising because the changes in the distribution of caspase-3-proficient cells after treatment for 24 h markedly differed from those observed in parental cells.

DRB, a known inhibitor of cellular kinases involved in the RNA synthesis [te Poele et al., 1999], at a concentration inducing nucleolar segregation, did not affect the cell-cycle progression (Fig. 3).

As mentioned above, flow cytometric analyses revealed an accumulation of $G_2$/M cells in response to ROSC treatment. To confirm that cells were blocked in $G_2$-phase, but not during mitosis, cell cultures were fixed and stained with Hoechst 33258 (Fig. 4). In untreated controls a number of mitoses were detected. However, in ROSC-treated specimens mitoses were found only occasionally, whereas the proportion of enlarged cells representing $G_2$-phase cells markedly increased. Furthermore, as previously shown, CDK1 became heavily phosphorylated at two inhibitory sites (Thr14/Tyr15) (Fig. 5) confirming that cells were arrested at the $G_2$/M checkpoint.

## ROSC INHIBITS ITS MAJOR TARGETS IN A DOSE- AND TIME-DEPENDENT MANNER

We have recently reported that the effect of ROSC on the functional status of major cell-cycle regulators such as CDK2 or CDK7 strongly differs between cancers cells and is dose- and time-dependent [Węsierska-Gądek et al., 2009b]. Reduction of the numbers of living MCF-7 cells following treatment with lower doses of ROSC (up to 10 μM) was marginal over the 24 h period indicating that CDKs were still active. Exposure of MCF-7 cells to 20 μM ROSC resulted in the inactivation of its major targets in a time-dependent manner (Fig. 5). According to the predictions, phosphorylation of CDK2 at Thr160 was strongly diminished in ROSC-treated cells and was observed not until 15 h (Fig. 5). The kinase responsible for activation of CDK2 by phosphorylating its Thr160 within T-loop is CDK7, whose reduced phosphorylation at Ser164/Thr170 indicates one possible mechanism by which ROSC inhibits CDKs in cells (Fig. 5). Phosphorylation of retinoblastoma protein (pRb) at Ser780 was also decreased in ROSC-treated cells. Reduction of phosphorylated pRb form was observed after treatment for 15 and 24 h (Fig. 5) and was more pronounced considering the fact that total pRb levels increased with ROSC treatment (Fig. 5). Furthermore, ROSC at higher doses was necessary to reduce phosphorylation of Ser5 within the carboxy-terminal domain (CTD) of RNA pol II. We also analyzed phosphorylation of survivin at Thr34 that is known to be catalyzed by CDK1 [O'Connor et al., 2000]. ROSC diminished the site-specific phosphorylation of survivin and also reduced its cellular level in MCF-7 cells. Finally, we determined the effect of ROSC treatment on cellular concentrations of two cell-cycle regulators: cyclin D3 and MCM7. ROSC decreased the levels of both proteins in a time-dependent manner (Fig. 5). Unlike ROSC, DRB, a strong inhibitor of CDK9, did not diminish the site-specific phosphorylation of both examined proteins. Finally, interference with estrogen signaling pathway by 4-hydroxytamoxifen (OHT), a selective estrogen response modifier (SERM) was determined. Treatment of MCF-7 cells with 0.5 μM OHT for 24 h had no effect on the modification and cellular levels of survivin and RNA pol II (Fig. 5).

## ACTIVATION OF wt P53 PROTEIN IN MCF-7 CELLS BY PHOSPHORYLATION OF Ser46

The p53 tumor suppressor is one of the key factors determining the $G_2$/M checkpoint. Therefore, we examined the changes in the

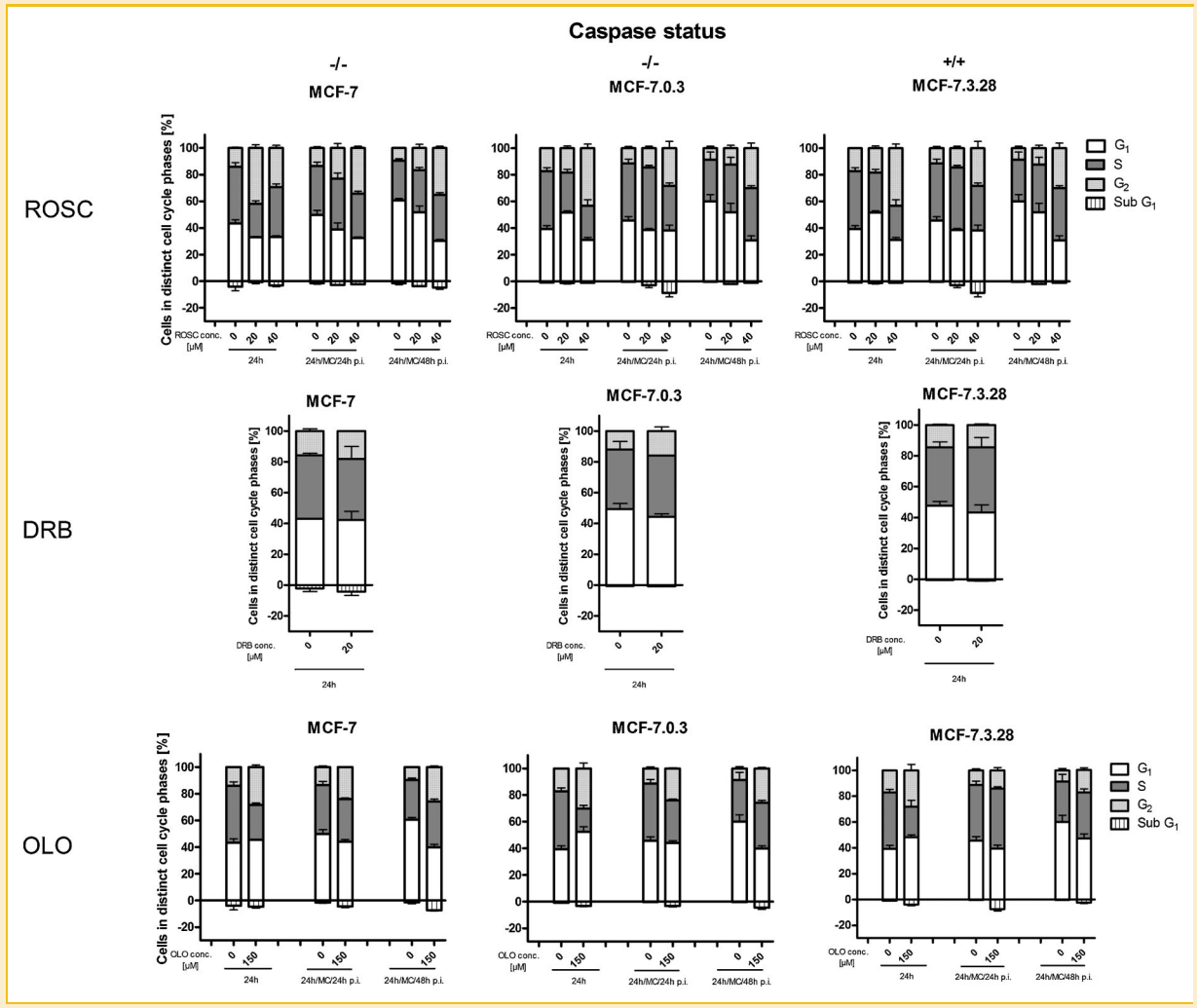


Fig. 3. Prolonged cell-cycle arrest induced in MCF-7 cells by ROSC. Parental MCF-7 cells transfected with empty CMV vector (MCF-7.0.3) and cells transfected with human caspase-3 (MCF-7.3.28) were treated with 20 μM ROSC, 40 μM ROSC, 20 μM DRB, and 150 μM OLO for 24 h. In some cases after treatment for 24 h medium was changed (MC) and after addition of a fresh drug-free medium cells were maintained in cell culture for 24 or 48 h. Cells were harvested and stained with propidium iodide. DNA concentrations were evaluated using ModFIT software. These data represent mean values ± SD from three independent experiments.

expression and modification of p53 protein in response to ROSC treatment. ROSC induced strong phosphorylation of p53 protein at Ser46 in parental MCF-7 cells and cells transfected with empty vector (MCF-7.0.3), but not in secondary mutant cells expressing caspase-3 (MCF-7.3.28), and the site-specific phosphorylation of p53 was associated with strong upregulation of total protein expression in MCF-7 cells (Fig. 6). In contrast, p53 protein concentration increased only weakly in cells reconstituted with caspase-3. It should be emphasized that both twin blots (with MCF-7 and MCF-7.3.28 samples) were processed in the same batch and were simultaneously exposed to film. Therefore, a significant difference in the signal intensity between the two cell lines reflects a real difference between them in p53 levels. Although a shorter film exposure offered better resolution of the p53 bands in samples obtained from parental cells, protein bands in MCF-7.3.28 were then barely detectable. Unlike ROSC, OLO weakly elevated cellular levels of p53 protein (data not shown). No increase of p53 concentration was detected after treatment with 20 μM DRB (data not shown). Remarkably, the strong upregulation of p53 in cells lacking caspase-3 correlated with an appearance of an 89 kDa fragment of PARP-1 representing its shortened form generated by activated effector caspases. Sequential incubation of the blots with anti-caspase-3 antibodies unequivocally evidenced that the strong activation of p53 by its phosphorylation at Ser46 occurs in MCF-7 cells lacking caspase-3. The strong induction of p53 tumor suppressor protein in ROSC-treated MCF-7 cells might be responsible for their rapid accumulation at the $G_2$/M checkpoint.

## INDUCTION OF p53AIP1 PROTEIN CORRELATES WITH PHOSPHORYLATION OF p53 AT Ser46

ROSC not only elevated the nuclear level of p53 protein but also activated its transcriptional activity, as demonstrated by observed changes in levels of $p21^{waf1}$ and MDM-2 proteins. The low basal level of $p21^{waf1}$ was elevated by ROSC after 4 h and increased further

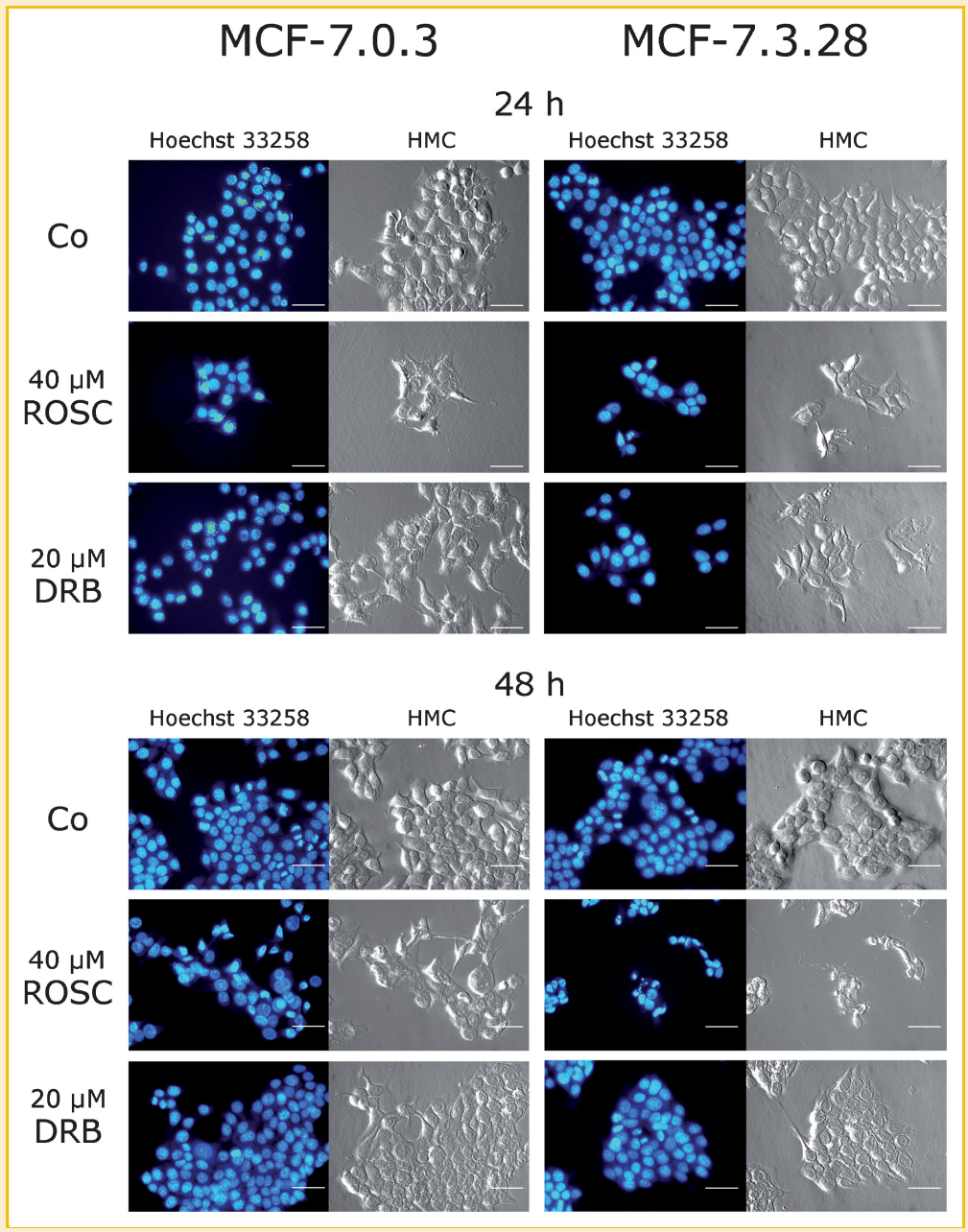


Fig. 4. Monitoring of changes in chromatin structure in cells after treatment with CDK inhibitors. Untreated controls and cells treated with 40 µM ROSC and 20 µM DRB for 24 h and for 48 h were fixed and stained with Hoechst 33258. Cell density of monolayer, cell size and phenotype were inspected by light microscopy using Hoffman Modulation Contrast (HMC) and chromatin structure visualized by Hoechst 33258 staining was monitored by fluorescence microscopy. White bars represent 50 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

at 8 h (not shown). To assess the functional consequences of the p53 activation by site-specific phosphorylation, we monitored levels of p53AIP1, a known transcriptional target of P-Ser46-p53 [Oda et al., 2000], in ROSC-treated MCF-7 cells and controls. In untreated controls p53AIP1 protein was undetectable in WCLs and isolated mitochondria. However, after ROSC treatment for 6 h p53AIP1 appeared in WCLs prepared from MCF-7 cells (Fig. 7), but not in samples obtained from caspase-3 reconstituted MCF-7.3.28 cells (Fig. 7). The antibodies recognized in mother cell line the splice variants of p53AIP1 protein. Thus, the expression pattern of p53AIP1 closely correlates with the phosphorylation of p53 at Ser46.

### ROSC STRONGER INDUCES CHANGES OF THE MITOCHONDRIAL MEMBRANE POTENTIAL IN CASPASE-3-DEFICIENT CELLS

To assess the impact of ROSC treatment on the membrane potential of mitochondria, we monitored the membrane potential using an electrochromic dye JC-1 [Reers et al., 1991]. JC-1, a positive charged

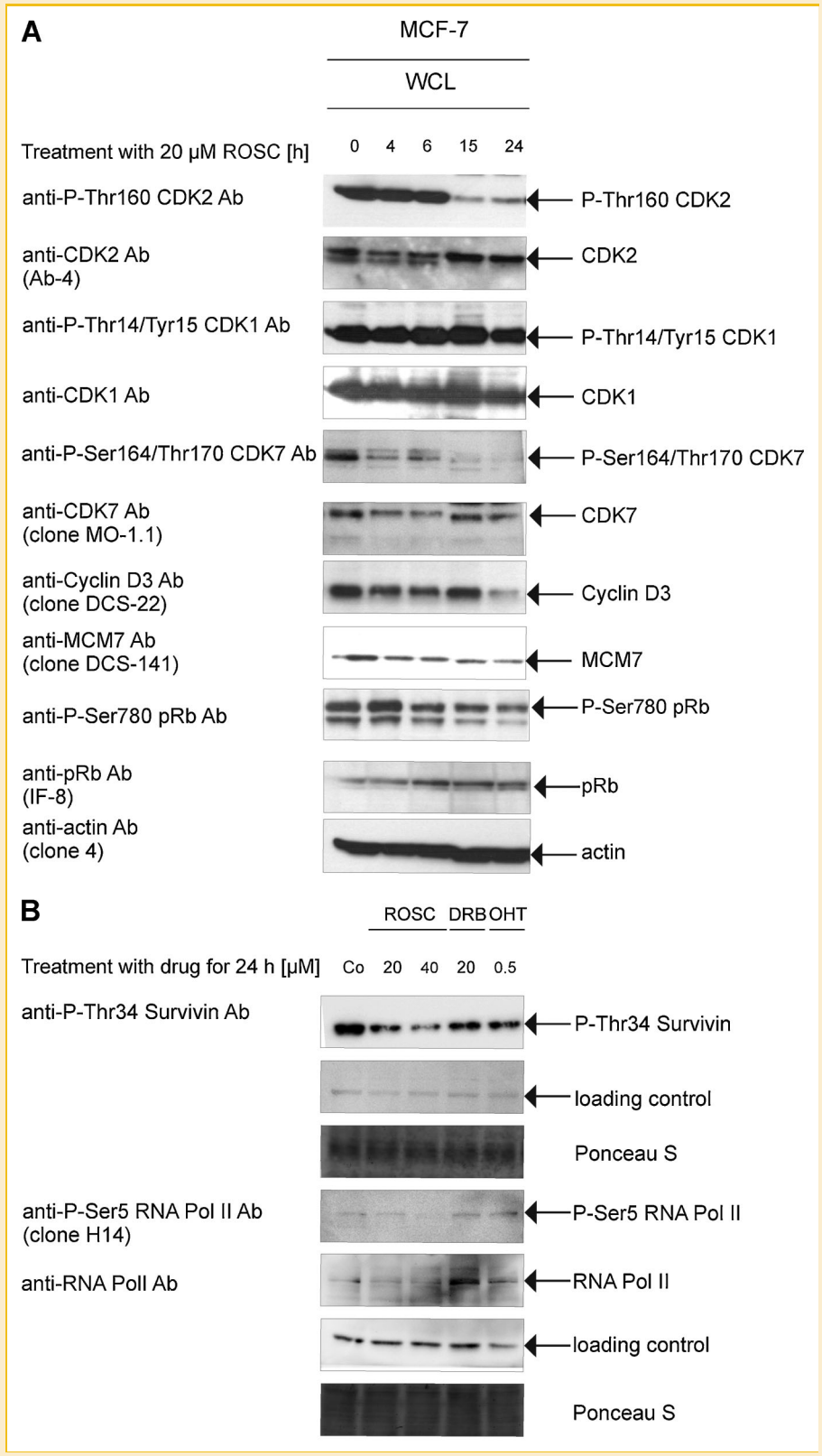


Fig. 5. ROSC negatively affects cell-cycle regulators in a time-dependent manner. Whole cell lysates (WCLs) prepared from untreated control cells and cells exposed to CDK inhibitors (ROSC and DRB) were separated on 12% or 15% SDS gels (30 μg/lane) and transferred onto a PVDF membrane. The blots were incubated with indicated primary antibodies in an appropriate concentration. After incubation with secondary antibodies linked to HRP, immune complexes were detected by chemiluminescence using ECL+. Equal protein loading was confirmed by Ponceau S staining of the membrane and by incubation with anti-actin antibodies. In Figure 4B WCL sample prepared from 4-hydroxytamoxifen (4-OHT)-treated cells was additionally loaded.

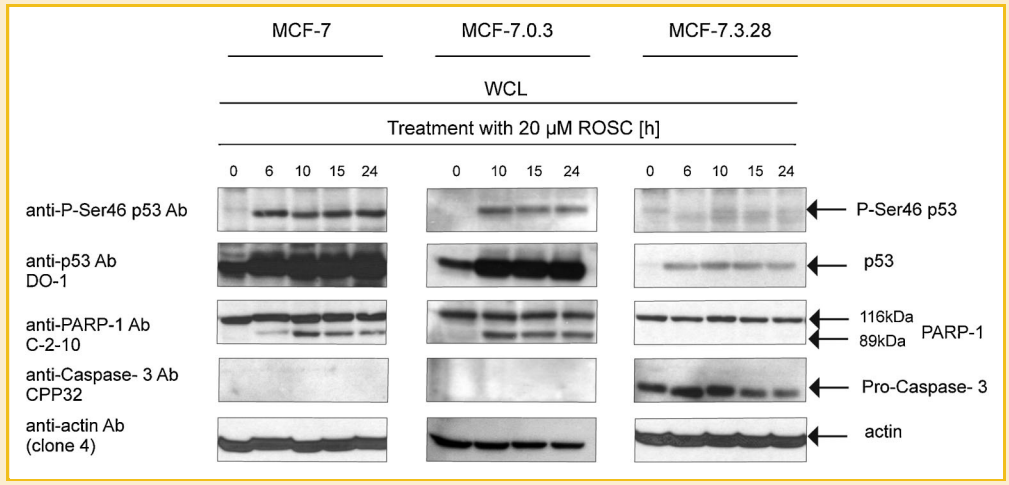


Fig. 6. ROSC induces phosphorylation of p53-Ser46 in MCF-7 cells. WCLs prepared from untreated control cells and cells exposed to ROSC were separated on a 10% SDS gel (30 μg/lane) and transferred onto a PVDF membrane. The blots were incubated with indicated primary antibodies in an appropriate concentration, then the site-specific phosphorylation of p53 was determined using affinity-purified anti-P-Ser46-p53 antibodies. Total p53 protein, in all forms, was detected after incubation with monoclonal anti-p53 DO-1 antibodies. The caspase-3 status of the samples was checked by sequential incubation with anti-caspase-3 antibodies. After incubation with secondary antibodies linked to HRP, immune complexes were detected by chemiluminescence using ECL+. Equal protein loading was confirmed by Ponceau S staining of the membrane and by incubation with anti-actin.

carbocyanine redistributes between compartments according to the membrane potential. Untreated controls and cells treated with drugs for 24 h were detached from the substratum by limited trypsinization and then incubated with JC-1. After cautious washing, cells were immediately analyzed by flow cytometry. As shown in Figure 8, almost all untreated control cells formed J-aggregates that generate red fluorescence. After exposure to 20 μM ROSC for 24 h several cells lost the capability to aggregate JC-1 in mitochondria and monomeric dye generating green fluorescent signals were accumulated in the cytosol (Fig. 8). Treatment of caspase-deficient breast cancer cells (MCF-7 and MCF-7.0.3) with 40 μM ROSC resulted in a loss of the potential of mitochondrial membrane in approximately 30% cells. Caspase-proficient cells (MCF-7.3.28) were less sensitive to the action of ROSC. Disruption of the potential of the mitochondrial membrane was detected in approximately 10% of cells. These results are in concordance with our previous observations [Wesierska-Gadek et al., 2005a]. However, treatment with staurosporine (STAU), a strong cytotoxic kinases inhibitor, resulted in a loss of the potential of mitochondrial membrane in the majority of both caspase-3-deficient cells (MCF-7) and caspase-3-proficient cells (MCF-7.3.28) (Fig. 8). Similar effects were observed after exposure of the examined cell lines to CP or other DNA damaging agents (results not shown).

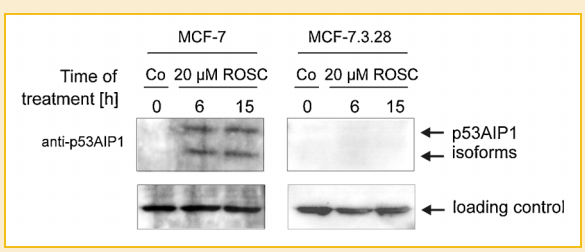


Fig. 7. Lack of induction of p53AIP1 protein in ROSC-treated caspase-proficient MCF-7.3.28 cells. Mitochondrial proteins prepared from MCF-7 and MCF-7.3.28 cells were resolved on 15% SDS gels. Proteins immobilized on membranes were incubated with antibodies as indicated. The unspecific band at about 50 kDa generated by polyclonal anti-p53AIP1 antibody is shown as loading control. The anti-p53AIP1 antibodies recognize two splicing variants.

## EXPOSURE TO ROSC DOES NOT INCREASE APOPTOTIC FRAGMENTATION OF NUCLEI IN CASPASE-3-PROFICIENT CELLS

It has been shown that caspase-3 is indispensable for morphological changes of chromatin associated with apoptosis [Janicke et al., 1998b]. Exposure of various human cancer cells, for example, HL-60 promyelocytic leukemia or HeLa cervix carcinoma cells, to ROSC initiates caspase-dependent apoptosis associated with an accumulation of hypoploid cells because of caspase-3-mediated activation of DNAse (CAD), DNA degradation and, hence, fragmentation of chromatin. It could be postulated that after reconstitution with caspase-3 MCF-7 breast cancer cells should respond to ROSC in a similar fashion. However, the DNA histograms indicated that the frequencies of hypoploid cells did not significantly differ between cells with and without caspase-3 following ROSC treatment. After treating parental MCF-7 cells with ROSC for 24 h a very low number of sub-$G_1$ cells appeared directly upon treatment or after washout followed by post-incubation (Fig. 3). Reconstitution of cells with caspase-3 did not increase the ratio of sub-$G_1$ cells representing apoptotic cell populations with fragmented chromatin. Although these results provided important indications that the outcome of ROSC treatment in MCF-7 cells does not depend on caspase-3 status, we also examined *in situ* chromatin changes in the cell cultures following ROSC treatment. After fixation with paraformaldehyde, untreated control cells and cells treated with ROSC and DRB for 24 and 48 h were stained with Hoechst 33258. As shown in Figure 4, at the time of starvation untreated control cells had a high mitotic

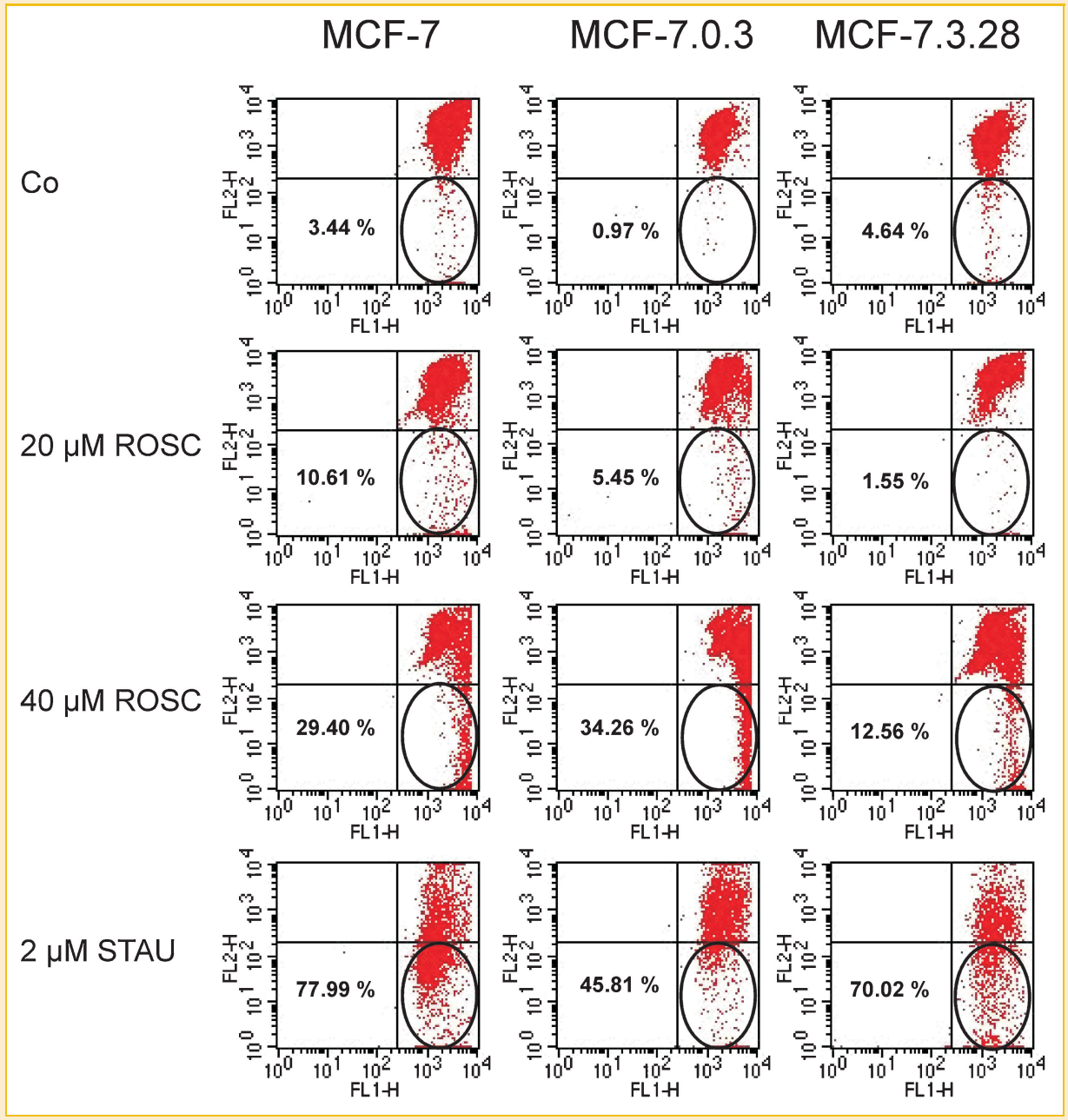


Fig. 8. ROSC-induced disruption of the mitochondrial transmembrane potential in human MCF-7 breast cancer cells. Controls and cells treated with 20 μM ROSC, 40 μM ROSC, or with 2 μM staurosporine (STAU) were incubated with JC-1 and directly after washing were analyzed by flow cytometry. Diagrams show two-color analysis revealed by flow cytometric measurement using the FITC channel for green monomers (Ex/Em = 510/527) and the PI channel for red J-aggregates (Ex/Em = 585/590). The population of cells that lost the capability to aggregate JC-1 is in low right quadrant (the numbers (% of gated cells) were included in left quadrant). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

potential. In parental caspase-3-deficient cells chromatin condensation was only observed in a low proportion of examined nuclei following treatment with ROSC, and even less frequently after exposure to DRB. Moreover, the proportion of nuclei displaying the characteristic condensed chromatin did not increase after longer treatment (Fig. 4). Surprisingly, the reconstitution of cells with caspase-3 neither increased the incidence of apoptosis nor generated cells with fragmented chromatin. In fixed cell preparations fragmentation of nuclei was detected only occasionally in MCF-7.3.28 cells, even after continuous treatment with ROSC at a higher concentration (Fig. 4).

### ROSC WEAKLY ACTIVATES PRO-APOPTOTIC FACTORS IN CASPASE-3-PROFICIENT MCF-7.3.28 CELLS

Incubation of blots with anti-PARP-1 antibodies unexpectedly showed that the antigen was specifically cleaved after ROSC

treatment to a large 89 kDa fragment in parental and MCF-7.0.3 cells, but not in caspase-3-proficient MCF-7.3.28 cells (Fig. 6). The full-length antigen and its truncated fragment were stained much more strongly in nuclear samples, confirming the specificity of the immune recognition (not shown). The PARP-1 cleavage began 6 h after onset of ROSC treatment and continued during the following 18 h. After 24 h the intensity of the signal corresponding to the full-length enzyme decreased. To confirm that MCF-7.3.28 cells do express caspase-3, the same blots were sequentially incubated with anti-caspase-3 antibodies. The results of the immunoblotting confirmed the presence of pro-caspase-3 exclusively in secondary mutant MCF-7.3.28 cells. Furthermore, the intensity of the pro-caspase-3 band remained almost unchanged after treatment, and cleaved subunits were not detected on the blot, even after prolonged exposure.

These observations raised two questions. Firstly, how can PARP-1 be proteolytically processed to an apoptotic 89 kDa fragment in cells lacking caspase-3? Secondly, why is caspase-3 not activated in MCF-7.3.28 cells in response to ROSC treatment? Therefore, in the next experimental series, we analyzed selected cellular factors that are involved in apoptosis in WCLs and subcellular fractions prepared from both control cells and cells exposed to ROSC. To avoid artificial damage of mitochondrial membranes during the isolation procedure, cells were gently fractionated according to the method described by Fiskum et al. [2000]. Effects of ROSC on the functional status of four caspases in MCF-7.3.28 cells were then determined by immunoblotting. WCLs were loaded on 15% SDS-slab gels to adequately resolve pro-caspases and their subunits that appear after pro-zymogen cleavage. As shown in Figure 9, low proportions of effector caspases -3 and -7 were cleaved after treatment, generating p20 and p17 subunits, after 15 and 24 h, respectively. The small subunit of caspase-8 (p18) was detected after 24 h. The intensity of the pro-zymogen bands remained almost unchanged, indicating that only small proportions of the caspases were processed (Fig. 9). Inductor caspase was hardly detectable in WCLs, therefore it was analyzed in the cytosol fraction, in which the intensity of the pro-caspase-9 band was not diminished after treatment with ROSC. In contrast to weak processing of pro-caspases-3, -7, and -8 in ROSC-treated proficient MCF-7.3.28 cells is proteolytic cleavage of apoptotic proteases observed in human leukemia cells after treatment with etoposide (Fig. 1).

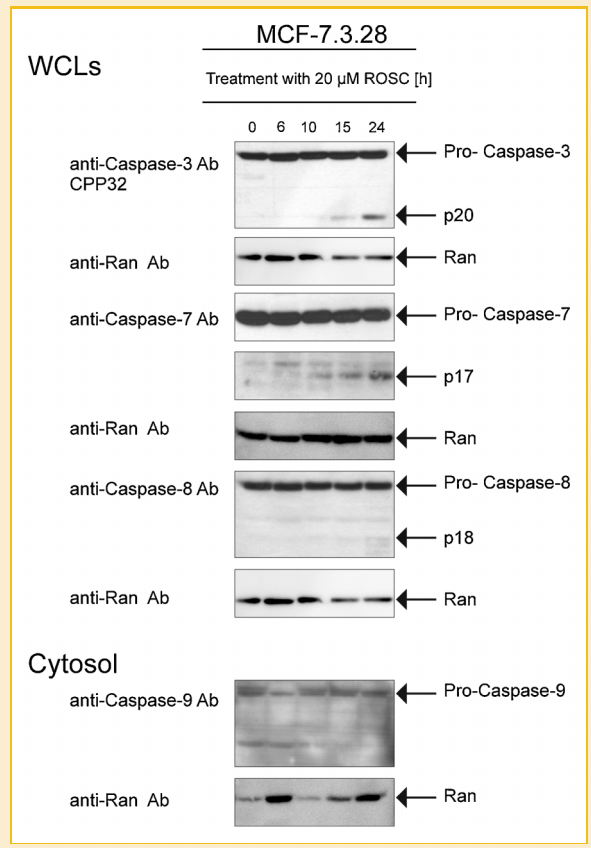


Fig. 9. Weak processing of caspases in caspase-3-proficient MCF-7 cells. WCLs prepared from untreated controls and cells treated with 20 μM ROSC for indicated periods of time were resolved on 15% SDS gels and transferred onto a PVDF membrane. Blots were incubated with indicated primary antibodies. Position of protein bands representing pro-caspases and their subunits are indicated. Transfer and immunoblotting conditions as described in detail in the legend to Figure 4.

## CDK INHIBITORS WEAKLY ACTIVATED CASPASES IN MCF-7 CELLS RECONSTITUTED WITH CASPASE-3

The results of the analyses of cellular regulators of apoptosis in MCF-7.3.28 cells indicated that ROSC failed to activate the caspase-dependent apoptotic pathway in them, despite the presence of caspase-3. To substantiate these observations, we finally determined the activity of caspase-3 in secondary mutant cancer cells. As controls, MCF-7.0.3 cells transfected with empty vector were included in these assays. To eliminate a potential risk of artifact generation, we used the cell-based caspase-3-GLO assay. We also determined the numbers of living cells in the samples in the same microtiter plate, and the measured enzyme activity was normalized against cell number. After treatment with ROSC at low concentrations (10 and 20 μM) the caspase-3 activity in MCF-7.3.28 was slightly lower than the basal level estimated in the control (Fig. 10). At a final ROSC concentration of 40 μM the activity of caspase-3 increased by approximately 150% and after treatment with DRB by 50%. The weak elevation of caspase-3 activity is not significant; ROSC at this dose usually, for example, in human HeLa or HL-60 cells [Komina and Wesierska-Gadek, 2008; Węsierska-Gądek et al., 2009b] induces the effector caspase by 1,000% or more. Surprisingly, neither ROSC nor DRB affected the activity of caspase-9.

## STRONG INDUCTION OF THE SITE-SPECIFIC PHOSPHORYLATION OF p53 AND CASPASE-3 ACTIVATION IN MCF-7.3.28 CELLS UPON TREATMENT WITH DNA DAMAGING AGENTS

Finally, we determined the activity of both caspases in two MCF-7 cell lines after treatment with STAU, a strong cytotoxic agent inducing apoptosis [Bruno et al., 1992]. STAU strongly activated both caspases exclusively in caspase-3-proficient cells. Interestingly, much stronger increase of caspase-3 activity (ninefold) was observed after treatment with 40 μM CP (Fig. 10) and alkylating agents (data not shown). The increase was statistically very highly significant as compared to the control cells as well as to the cells

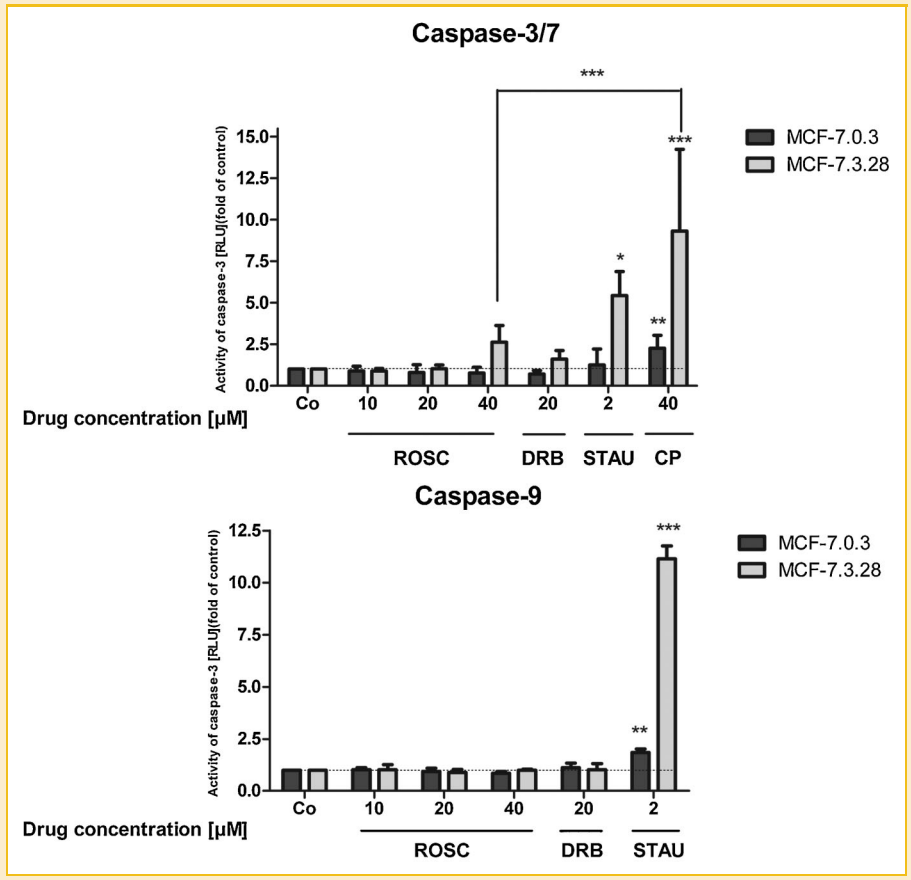


Fig. 10. DNA damaging agents strongly induce activity of caspases in caspase-3-proficient MCF-7 cells. Exponentially growing MCF-7.0.3 and MCF-7.3.28 cells were treated in a multiwell plate for 24 h with drugs at indicated concentrations. The activities of cellular caspase-3/7 and caspase-9 were determined in quadruplicate using the Caspase-3-GLO and Caspase-9-GLO Assays. The activity of the caspases [relative luminescence units (RLU)] ± SD was normalized to the numbers of viable cells, determined by CellTiterGlo Assays.

exposed to 40 μM ROSC (Fig. 10). These observations might indicate that activation of caspases in secondary mutant MCF-7 cells expressing caspase-3 requires a strong signaling, for example, after DNA damaging. To check it, we analyzed WCLs from control cells and cells treated with 40 μM CP [Silver et al., 2010] for 15 and 24 h. As shown in Figure 11A, a very weak processing of pro-caspases-7 occurred after CP treatment for 15 h in the mother cells but not in cells reconstituted with caspase-3 (Fig. 11A). In the latter cell line the strong processing of effector pro-caspases (3 and 7) was detected after CP treatment for 24 h. Interestingly, the late onset of proteolytic cleavage of pro-zymogens coincided with the delayed induction of p53 protein in caspase-3-proficient MCF-7 cells. Remarkably, in mother cell line a very strong upregulation of p53 protein occurred after treatment with CP for 15 h, whereas in caspase-3 reconstituted cells p53 reached the comparable level after treatment with CP for 24 h. Unlike ROSC and OLO, STAU, and CP induced a strong DNA damage and activated the cellular signaling pathway resulting in a strong phosphorylation of γ-H2AX at Ser139 (data not shown) and in turn the phosphorylation of p53 at Ser15 (Fig. 11B). The site-specific phosphorylation of p53 was detected in both caspase-3 negative and positive MCF-7 cells.

These data are consistent with the results of other experiments and provide further evidence that the reconstitution of caspase-3 in MCF-7 cells does not reprogram their apoptotic responses to treatment by agents like selective CDK inhibitors that do not impair DNA.

## DISCUSSION

Human MCF-7 breast carcinoma cells display a number of alterations in cell cycle, signal transduction and apoptosis pathways, several of which render them insensitive to a number of anti-cancer drugs, including: upregulation of ER-α; inactivation of the cellular INK4A gene encoding both $p16^{INK4A}$ protein (a cellular CDK inhibitor) and $p14^{ARF}$ protein (which regulates the interaction between MDM2 and wt p53 tumor suppressor protein); and loss of caspase-3 activity due to a 47-bp deletion within exon 3 of the *caspase-3* gene [Fan et al., 1995; Devarajan et al., 2002].

Therefore, much higher doses of conventional chemotherapeutics are required to stop proliferation of exponentially growing MCF-7 cells. Unfortunately, most classic cytostatic drugs, especially when

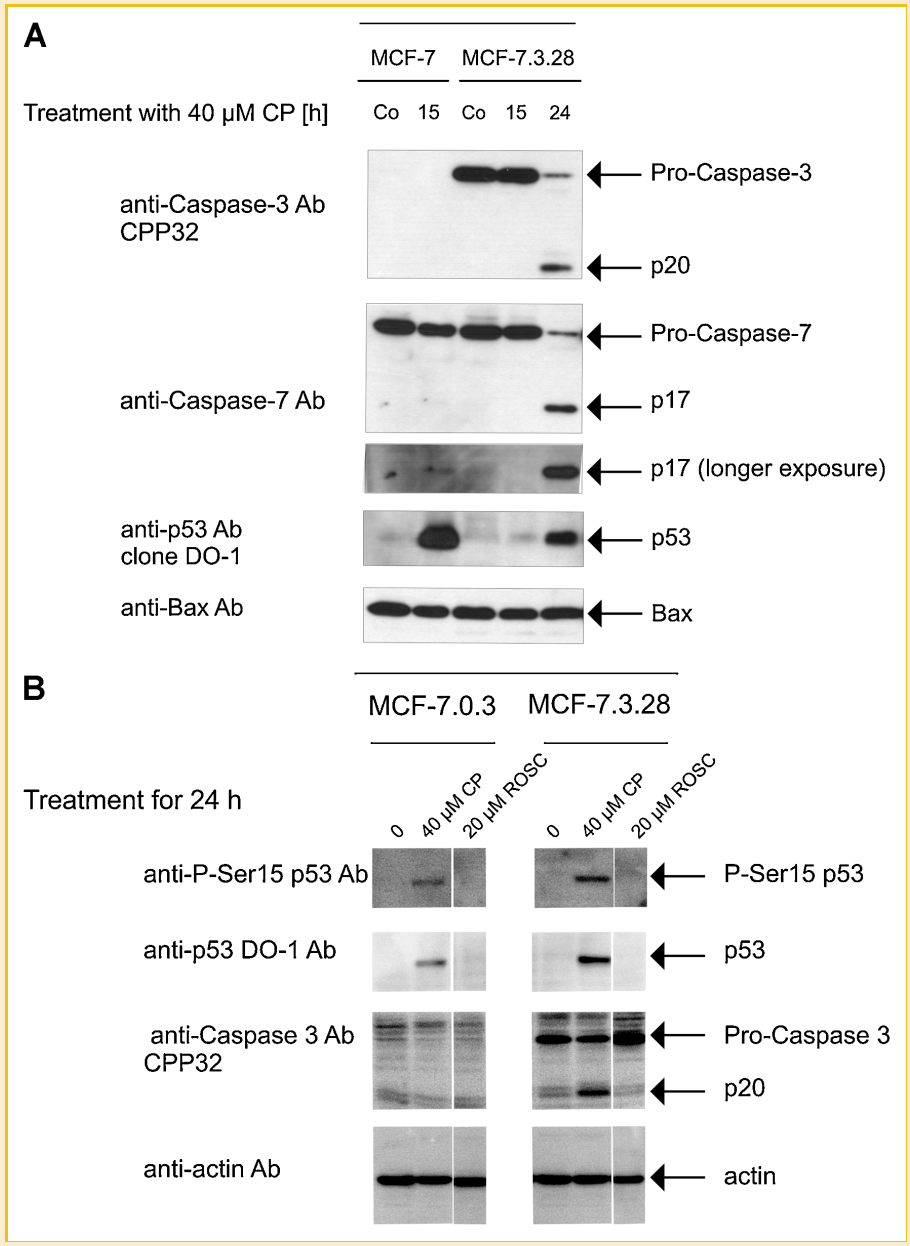


Fig. 11. Activation of p53 protein and effector caspases upon DNA damage. WCLs prepared from untreated controls and cells treated with 40 μM CP or with 20 μM ROSC for indicated periods of time were resolved on 15% SDS gels and transferred onto a PVDF membrane. Blots were incubated with indicated primary antibodies directed against caspases and antibodies directed against P-Ser15p53 and total p53 protein. Position of pro-caspases and their fragments generated during processing are indicated.

applied at higher dosage, result in severe DNA damage and hence may induce development of a secondary malignancy. Therefore, the development and application of alternative, non-genotoxic drugs would be advantageous. Pharmacological inhibitors of CDKs fulfill these criteria and might substitute deficits in the proper regulation of the cell cycle [Wesierska-Gadek and Krystof, 2009; Wesierska-Gadek et al., 2010]. Furthermore, MCF-7 breast cancer cells harbor an intact p53 pathway, rendering them sensitive to agents (re)activating p53 responses. Therefore, it seems reasonable to hypothesize that selective pharmacological inhibitors of CDKs that simultaneously (re)activate p53 tumor suppressor protein could be effective therapeutic agents for inhibiting both the proliferation and survival of human MCF-7 breast cancer cells.

During the last two decades a number of selective CDK inhibitors have been developed, and approximately 20 have been proved to be effective in experimental tests and qualified for clinical trials [Wesierska-Gadek and Schmid, 2006; Wesierska-Gadek and Krystof, 2009]. ROSC, a tri-substituted purine derivative (Seliciclib™; CYC-202) is one of the most promising compounds that is now in advanced stages of clinical testing. Although it inhibits several kinases, CDK2 and CDK7 are its major targets. Biological effects of ROSC strongly depend on cell type, the constellation, and functional

status of a variety of cellular factors regulating signal transduction and apoptosis, the concentration applied and duration of treatment. It is generally accepted that at low concentrations ROSC blocks cell-cycle progression and at higher doses induces programmed cell death [Wesierska-Gadek et al., 2004a, 2005a, 2009a]. This duality of ROSC action is attributable to differential targeting of its major downstream players. At low doses it effectively inhibits CDK2 in MCF-7 cells, inducing cell-cycle arrest in asynchronously growing cells with high mitotic index [David-Pfeuty, 1999; Schmid et al., 2005; Wesierska-Gadek et al., 2005a]. However, the impact of ROSC markedly changes when synchronized cells are treated [Maurer et al., 2009; Węsierska-Gądek et al., 2009b]. Higher concentrations of ROSC negatively affect the activity of CDK7, and in turn, the phosphorylation of Ser5 residues within the repetitive motif of RNA pol II localized in the CTD. Abolishment of this modification, and hence the next processing step at Ser2, catalyzed by CDK9, prevents the recruitment of specific cofactors by RNA pol II that are required for the switch from initiation to elongation of transcription. Thus, the inhibition of phosphorylation of CDK7 at Ser164/170 by ROSC results in a block of global transcription. The outcome of the impairment of transcription largely depends on the type of cells, cellular context, and functional status of the major players in the signal transduction and apoptosis pathways. Thus, for instance, in human HeLa cervix carcinoma cells ROSC represses HPV-encoded E6 and E7 oncoproteins, thereby restoring the $G_1$/S checkpoint and facilitating induction of apoptosis [Wesierska-Gadek et al., 2008a, 2009b]. Remarkably, by abolishing the site-specific phosphorylation ROSC changes the functional status of multiple cellular proteins. *Inter alia*, it can: prevent phosphorylation of survivin at Thr34, resulting in its destabilization and downregulation during the execution of apoptosis in HeLa cells; diminish phosphorylation of Bad at Ser112 and activate the protein by releasing it from its complex with 14-3-3 protein; and repress the E6 oncoprotein, resulting in the escape of wtp53 protein from rapid ubiquitylation and degradation in proteasomes; reactivated p53 tumor suppressor has then been shown to induce cell-cycle block and apoptosis at different stages [Wesierska-Gadek et al., 2008c, 2009b].

Due to its pleiotropic effects, ROSC is very efficient not only against rapidly dividing cancer cells, but also against resting cells like lymphocytes circulating in the peripheral blood of patients with CLL. The interference with transcriptional elongation in resting cells like CLL or multiple myeloma cells primarily affects the cellular levels of aberrantly overexpressed anti-apoptotic proteins, thereby facilitating the initiation and execution of apoptosis [Paprskarova et al., 2009; Rogalinska et al., 2010].

Interestingly, ROSC is also capable of inducing apoptosis in human MCF-7 breast cancer cells despite their lack of caspase-3 [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2008b, 2009a]. ROSC very rapidly arrests cell-cycle progression, resulting in an accumulation of $G_2$ cells. Even after treatment for 6 h, approximately 50% of human MCF-7 cells are blocked in $G_2$ phase [Wesierska-Gadek et al., 2006]. Interestingly, 3 h after onset of treatment ROSC induces phosphorylation of p53 tumor suppressor protein at Ser46 catalyzed by HIPK2, resulting in a transcriptional induction of p53AIP1 protein [Wesierska-Gadek et al., 2005a, 2007]. This protein, after *de novo* synthesis and translocation into mitochondria promotes dissipation of the potential of the mitochondrial membrane and, consequently, the release of some mitochondrial proteins, for example, AIF, APAF-1, and cytochrome *c* into the cytosol, thereby inducing apoptosis. However, the course of programmed cell death initiated in MCF-7 cells by ROSC is atypical, because in the absence of caspase-3 some important steps during execution of apoptosis, for example, the activation of caspase-dependent DNA-se (CAD), DNA cleavage and breakdown of chromatin do not occur [Janicke et al., 1998b]. Therefore, one would expect the reconstitution of caspase-3 to facilitate apoptosis and restore its execution, with typical characteristics including activation of caspase-3 and its downstream effects (DNA cleavage, chromatin fragmentation, etc.). Accordingly, increased sensitivity of caspase-3 reconstituted MCF-7 to certain chemotherapeutic agents has been reported by a few groups [Janicke et al., 1998a; Yang et al., 2001]. However, contrary to expectations, reconstitution of MCF-7 cells with caspase-3 did not render them susceptible to the action of agents like ROSC and OLO that did not damage DNA. Neither cell-cycle block nor the apoptosis rate increased in proficient MCF-7.3.28 cells after treatment with CDK inhibitors.

In attempts to explain these results it should first be noted that in parental human MCF-7 cancer cells wt p53 protein is the major factor determining the apoptotic response to ROSC. Unlike in parental cells, in MCF-7.3.28 cells the p53 response to ROSC was much weaker. Remarkably, no phosphorylation of p53 protein at Ser46 was detected in caspase-3-proficient MCF-7 cells. This seems to be linked to the caspase-3 expression rather than to the transfection per se, because in MCF-7 cells transfected with an empty vector the site-specific phosphorylation of p53 was detected. Considering the dominant role of wt p53 in the induction and regulation of programmed cell death at its distinct stages, much curbed apoptotic response of MCF-7-proficient cells to ROSC might be, at least partially, related to weaker activation of p53 pathway.

It has been reported previously that ROSC-activated p53 through inhibition of RNA synthesis [Ljungman and Paulsen, 2001] and downregulation of MDM-2 expression at both mRNA and protein levels [Lu et al., 2001]. However, our results do not support these observations, for several reasons. First, we found strong enhancement of MDM-2 protein in MCF-7 cells after ROSC treatment. Furthermore, if the ROSC-mediated suppression of MDM-2 was a general phenomenon, one would expect ROSC to have similar effects on p53 in both MCF-7 cell lines, irrespective of their caspase-3 status. However, we cannot exclude the possibility that other factors, such as $p14^{ARF}$ protein, are also involved in the interplay between p53 and MDM-2 proteins after treatment with CDK inhibitors. The *p53AIP1* gene, a target for the tumor suppressor p53, is inducible by Ser46-phosphorylated p53 [Oda et al., 2000]. The *p53AIP1* gene generates three transcripts (α, β, and γ) by alternative splicing, encoding peptides of 124, 86, and 108 amino acids, respectively. Since p53AIP1α and p53AIP1β are localized in mitochondria they are potential mediators of mitochondrial membrane potential depolarization. Accordingly, it has been shown that ectopic overexpression of p53AIP1 [Oda et al., 2000] can induce apoptosis, and adenovirus-mediated *p53AIP1* gene transfer can enhance elimination of p53-resistant tumor cells by increasing the apoptosis rate [Yoshida et al., 2004].

Unlike ROSC, CP activates p53 in both parental MCF-7 and caspase-3-proficient MF-7.3.28 cells. CP generates DNA damage and in consequence induces in human MCF-7 breast cancer cells a cellular signaling pathway resulting in phosphorylation of p53 at Ser15 and its stabilization. We have previously reported that DNA damaging drugs like CP or alkylating agents induce signaling cascade by activation of Chk2, NBS1 leading to phosphorylation of H2A.X at Ser139 in human HeLa and MCF-7 cells and that activation of signaling pathway preceded activation of caspases-3 in HeLa cells [Wesierska-Gadek et al., 2008b]. Moreover, DNA damaging agents like STAU or CP strongly enhance processing of caspases and induce their activity in both caspase-3-deficient and proficient MCF-7 cells. However, caspase-3-proficient human MCF-7 cells benefit from the reconstitution solely when exposed to DNA-damaging drugs. Caspase-3 reconstitution sensitizes MCF-7.3.28 to the action of DNA-damaging agents by increasing caspase-dependent apoptosis.

## ACKNOWLEDGMENTS

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