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Interference With ER-α Enhances the Therapeutic Efficacy of the Selective CDK Inhibitor Roscovitine Towards ER-Positive Breast Cancer Cells

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ABSTRACT

In recent years many risk factors for the development of breast cancer that are linked to estrogens have been identified, and roscovitine (ROSC), a selective cyclin-dependent kinase (CDK) inhibitor, has been shown to be an efficient inhibitor of the proliferation of human breast cancer cells. Therefore, we have examined the possibility that interference with estrogen signaling pathways, using tamoxifen (TAM), a selective estrogen receptor modulator (SERM), could modulate the efficacy of treatment with ROSC. In conjunction with TAM, ROSC exhibited enhanced anti-proliferative activity and CDK inhibition, particularly in estrogen-dependent MCF-7 cells. The interaction between both drugs was synergistic. However, in ER-α-negative cells the interaction was antagonistic. Exposure of MCF-7 cells to ROSC abolished the activating phosphorylation of CDK2 and CDK7 at $Ser^{164/170}$. This in turn prevented the phosphorylation of the carboxyl-terminal repeat domain of RNA Polymerase II and ER-α at Ser^{118}, resulting in the down-regulation of the latter. Concomitantly, wt p53 was strongly activated by phosphorylation at Ser^{46}. Our results demonstrate that ROSC negatively affects the functional status of ER-α, making it potentially useful in the treatment of estrogen-dependent breast cancer cells. J. Cell. Biochem. 112: 1103–1117, 2011.

KEY WORDS: ER-α ACTIVATION; P-SER^{118}-ER-α; ESTROGEN; G_2 ARREST; CELL CYCLE; ACTIVATION OF WT P53; ANTI-ESTROGEN; TRANSCRIPTIONAL ELONGATION; SERMs

Breast cancer is the most frequently observed malignancy in women and is the second most common cancer-related cause of death among women worldwide [Glass et al., 2007]. Its prevalence increases with age, and the incidence of breast cancer varies approximately 4- to 5-fold between Western and less developed countries [Murray and Lopez, 1997; Key et al., 2001].

It is widely accepted that breast cancer is a complex disease, arising from the presence of specific alleles and the interplay between them, and from environmental factors that seem to determine the accumulation of mutations in essential genes [Ford et al., 1994; Hulka and Stark, 1995; Peto et al., 1999; Nathanson et al., 2001]. In recent years, numerous risk factors for the development of breast cancer have been identified, many of which are linked to estrogens [Hilakivi-Clarke, 2000; Russo and Russo, 2006; Russo et al., 2006; Russo and Russo, 2008], and diverse findings have implicated estrogens and/or other steroids in its initiation and/or progression, including the following. Epidemiological data suggest that endocrine factors play a major role in the etiology of the disease [Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Key and Verkasalo, 1999; Magnusson et al., 1999; Ross et al., 2000]. Steroid hormones are known to act *via* specific receptors in target tissues [for reviews, see Enmark and Gustafsson, 1999; Matthews and Gustafsson, 2003], thereby affecting the expression of genes that promote cell proliferation

Abbreviations used: CKI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; IAP, inhibitor of apoptosis protein; p53AIP-1, p53 apoptosis inducing protein 1; PARP-1, poly(ADP-ribose) polymerase-1; PCNA, proliferating cellular antigen; PD, Petri dish; PVDF, polyvinylidene difluoride; ROSC, roscovitine; WCL, whole-cell lysate; WT, wild-type.

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such as *CCND1* and/or other cellular targets regulating cell death [Wakeling et al., 1991; Nass and Dickson, 1997; Sabbah et al., 1999; Arnold and Papanikolaou, 2005; Hartman et al., 2009]. In addition, the animal experimental model established by Sicinski et al. [1995] provides evidence for a close functional link between the major G_1 cyclin and steroid hormone-regulated processes, and cyclin D_1 knock-out mice display defects in hormone-responsive proliferation of the epithelium during pregnancy [Sicinski et al., 1995].

According to a broadly accepted view, estrogens regulate proliferation and other processes in target tissues *via* transcriptional mechanisms involving the classic estrogen receptor (ER) [Walter et al., 1985]. ER-α, the major ER (a member of the steroid nuclear hormone receptor protein family [Mangelsdorf et al., 1995]), is a transcription factor [Katzenellenbogen and Katzenellenbogen, 1996] that has a number of splice variants, some of which influence the activity and function of the regularly spliced ER-α form [Hopp and Fuqua, 1998; Jazaeri et al., 1999]. Like other members of the superfamily, it has a modular structure that includes functional domains for ligand- and DNA-binding as well as two regions that modulate its transcriptional activity [Katzenellenbogen et al., 1996]. One of these regions, designated transcription activating function-1 (AF-1), is located in the NH_2-terminal part of the protein and functions in a ligand-independent manner. The other (AF-2) is located in the COOH– domain and is ligand-dependent. In addition, it is regulated by interaction with estrogen-responsive promoters and the recruitment of various cofactors to these promoters [Brzozowski et al., 1997]. The presence of two separate AFs facilitates fine-tuning of the receptor's activity [Brzozowski et al., 1997]. ER-α is a phosphoprotein, and its phosphorylation is strongly enhanced in response to ligand- and growth factor signaling pathways [Aronica and Katzenellenbogen, 1993; Kato et al., 1995]. It is phosphorylated at several residues by various protein kinases [Washburn et al., 1991; Ali et al., 1993; Lannigan, 2003], and its phosphorylation status in human breast cancer *in vivo* was recently determined [Murphy et al., 2009].

ER-α has several phosphorylation sites that seem to be important in the therapy and prognosis of breast cancer, including serines 104/106, 118, 167, and 305, which are modified by mitogen-activated protein kinases (MAPK), AKT, and p21Pak1 kinases, respectively. There are two highly conserved serine residues (Ser^{106} and Ser^{118}) in the amino-terminal AF-1 domain [Joel et al., 1995]. Estradiol has been found to trigger extensive phosphorylation of Ser^{118} [Joel et al., 1995]. Phosphorylation of this residue seems to be catalyzed by several cellular kinases [for a review, Lannigan, 2003]. Kato et al. [1995] reported that Ser^{118} is phosphorylated by MAPK *in vitro* and in COS-1 cells exposed to growth factors EGF and IGF in a ligand-independent manner. However, Joel et al. [1998] found that ligand-dependent phosphorylation of ER-α at the same serine residue seems to be catalyzed by cyclin-dependent kinase 7 (CDK7) [Chen et al., 2000]. Although Ser^{118} is located in the amino-terminal ligand-independent AF-1, it has been shown to be involved in the modulation of estrogen-induced ER-α activation. Mutation of Ser^{118} markedly reduces transactivation by ER-α [Ali et al., 1993; Le Goff et al., 1994; Kato et al., 1995].

In addition to elevated activation and/or overexpression of ER-α, other perturbations in cellular signaling pathways and/or the proper control of the cell cycle are often observed in human breast cancer [Sutherland and Musgrove, 2004]. These include the upregulation of cyclin D [Schuuring et al., 1992; Couse and Korach, 1999] and HER2 [Murphy and Fornier, 2010], and constitutive activation of the RAS signaling cascade, all of which affect cell cycle regulation, cell proliferation, and apoptosis [Nathanson et al., 2001]. The inactivation of cellular inhibitors of 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]. These defects in cell cycle regulation can be mitigated by applying pharmacological inhibitors of CDKs [Lapenna and Giordano, 2009; Sutherland and Musgrove, 2009]. In the last 20 years, a number of selective CDK inhibitors have been developed [Lapenna and Giordano, 2009; Węsierska-Gądek et al., 2009b; Galons et al., 2010; Rizzolio et al., 2010] Roscovitine (ROSC), a tri-substituted purine derivative (Selicliclib™; CYC-202), inhibits 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 the duration of the treatment [Wesierska-Gadek et al., 2009a]. We and other groups reported several years ago that ROSC efficiently inhibits the proliferation and cell cycle progression of chemoresistant human MCF-7 breast cancer cells [Wojciechowski et al., 2003]. It was found to arrest MCF-7 cells at the G_2/M phase of the cell cycle and concomitantly induce apoptosis. It has been found more recently that ROSC reduces the basal phosphorylation of ER-α and also prevents its ligand-induced activation [Wesierska-Gadek et al., 2011a]. In the present work, we sought to determine whether the use of tamoxifen (TAM), a selective estrogen response modifier (SERM), to interfere with ER-α might affect the efficacy of treatment with ROSC. Human ER-α-positive MCF-7 breast cancer cells and two ER-α-negative cell lines (BT-20 and SKBr-3) were used in the study. ROSC was effective towards all tested breast cancer cell lines. However, MCF-7 cells were much more sensitive to the action of ROSC than other cells. Our results indicate that ROSC affects estrogen signaling pathway by several distinct mechanisms and it acts synergistically in combination with TAM.

EXPERIMENTAL

DRUGS

The purine-derived CDK inhibitor ROSC was obtained from Prof. M. Strnad (Palacky University, Olomouc, Czech Republic). Staurosporine (STAU), 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), estradiol (E2), TAM, and 4-hydroxytamoxifen (4-HOT) were purchased from Sigma–Aldrich (St. Louis, MO). Stock solutions of the drugs in appropriate solvents were prepared. The solvents and concentrations employed were: ROSC (DMSO, 50 mM), DRB (DMSO, 50 mM), STAU (DMSO, 1 mM), TAM (ethanol, 1 mM), 4-HOT (ethanol, 5 mM), and E2 (DMEM, 10 μM). Aliquots of the stocks were stored at −20°C until use. Epidermal growth factor (EGF) was obtained from New England Biolabs (Beverly, MA).

CELLS AND TREATMENT

Human primary cancer cell lines were purchased from American Type Culture Collection. The following cell types were used: Human

MCF-7, BT-20, and SKBr-3 breast carcinoma cells, secondary mutant MCF-7-E6 cells expressing HPV-encoded E6 oncoprotein under control of the CMV promoter, and MCF-7 cells transfected with an empty vector. MCF-7 cells were grown as a monolayer in Dulbecco's medium, without phenol red, supplemented with 10% fetal calf serum (FCS) at 37°C in an 8% CO_2 atmosphere. SKBr-3 cells were cultivated in DMEM medium with 10% FCS and BT-20 cells in RPMI with 10% FCS. The secondary mutant MCF-7-E6 cell line and cells transfected with a control vector were maintained as previously described [Wesierska-Gadek et al., 2008]. Culture media were from (Sigma–Aldrich). Cells were grown to 60–70% confluence and then treated with ROSC at concentrations ranging from 1 to 40 μM for the periods of time indicated in Figures 2–5. ROSC, DRB, and STAU were dissolved as a stock solution in DMSO and stored at −20°C until use. In some experiments, MCF-7 cells were treated for 30 min with EGF at a final concentration of 10 nM.

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-Thr^{14}/Tyr^{15} CDK1, anti-phospho-Thr^{160} CDK2, anti-phospho-Ser^{780} pRb, anti-phospho-Ser^{474} AKT, and corresponding antibodies against the total antigen (all from New England Biolabs), polyclonal anti-phospho-Ser^{164}/Thr^{170} 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-Thr^{34} survivin, anti-pRb (IF-8), anti-PCNA mouse monoclonal antibody (PC-10) (all from Santa Cruz Biotechnology, CA), anti-CDK7 (clone MO-1.1), anti-ER-α (Sigma–Aldrich), rabbit monoclonal anti-phospho Ser^{118} ER-α (Epitomics, Burlingame, CA); anti-DBC 1, anti-RNA polymerase II phosphorylated on Ser-5 (clone H14), anti-RNA polymerase II phosphorylated on Ser-2 (clone H5) (all from Abcam plc, Cambridge,); and anti-RNA polymerase 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).

DETERMINATION OF NUMBERS OF LIVING CELLS

The numbers of viable human breast cancer cells and their sensitivity to the tested drugs at various concentrations were determined using CellTiter-Glo™ assays (Promega Corporation, Madison, WI). As described recently [Wesierska-Gadek et al., 2005b], the CellTiter-Glo™ luminescent cell viability assay measures luminescent signals, which are correlated with cellular ATP levels. Tests were performed at least in quadruplicate, and the cells' luminescence was measured using 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 (Figs. 2, 3, 6, and 7). The effects of the combined ROSC and TAM treatments on the IC_{50} values are shown in Figure 7 and are defined as reduction factors (reduction factor = IC_{50} ROSC/IC_{50} ROSC + TAM).

INTERACTION ANALYSIS BY THE CALCUSYN METHOD

Two methods of interaction analysis were used to determine whether the drug combination exhibited synergistic, additive, or antagonistic effects. The first was the combination index (CI) method of Chou and Talalay [1984]. CalcuSyn software (Version 2.0, Biosoft, Cambridge, UK), which is based on this method and takes into account both potency [median dose (Dm) or IC_{50}] and the shape of the dose–effect curve (the m-value), was used to calculate the CI. The program automatically graphs the data and produces reports of summary statistics for all of the drugs considered, together with a detailed analysis of drug interactions including the CI. A combination is considered to be synergistic if CI <1, additive if CI $= 1$, and antagonistic if CI > 1. For this analysis, data obtained on the effects of the combined ROSC and TAM treatments at each tested concentration. The fraction of cells affected and the corresponding CI values were calculated for each concentration.

MEASUREMENT OF DNA IN SINGLE CELLS BY FLOW CYTOMETRY

The DNA content of single cells was measured flow cytometrically, using the method of Vindelov [1977], with slight modifications as previously described [Wesierska-Gadek and Schmid, 2000]. Briefly, the adherent cells were detached from the substratum by limited trypsinization, then all cells were harvested by centrifugation and washed in PBS. Aliquots of 1×10^6 cells were stained with propidium iodide as previously described and 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 [Reers et al., 1991] (Molecular Probes Inc., Eugene, OR) as previously described [Kovar et al., 2000]. Control and treated cells were harvested, washed, and incubated with the dye at a final concentration of 10 μM for 5 min then extensively washed with PBS and immediately subjected to two-colour flow cytometric analysis. In intact cells JC-1 accumulates in the mitochondria as red fluorescent aggregates (J-aggregates, with excitation and emission maxima at 488 and 570 nm, respectively), but following mitochondrial depolarization it is present as green fluorescent monomers, with excitation and emission maxima at 488 and 530 nm, respectively.

ELECTROPHORETIC SEPARATION OF PROTEINS AND IMMUNOBLOTTING

Total cellular proteins dissolved in SDS sample buffer were separated on 10%, 12%, or 15% SDS slab gels, transferred electrophoretically onto polyvinylidene difluoride membrane [PVDF; GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England (formerly Amersham Biosciences)] 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 1% BSA in Tris-saline-Tween-20 (TST) buffer [Wesierska-Gadek et al., 2004]. 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 ANALYSES

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. 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. In the Tables and Figures such differences are indicated by three asterisks (***), two asterisks (**), one asterisk (*), and no asterisks, respectively. The statistical analysis of results in Figure 5 was performed using SPSS Statistic Base 17.0 software (SPSS Inc., Chicago, Il). Prior to the statistical analysis of the data, the ratios of cells not capable to aggregate JC-1 stain were logarithmically transformed. The analysis was performed using t-test. The reported *P*-value is a result of a two-sided test. A *P*-value 5% to 1% is considered statistically significant (*), 1% to 0.1% statistically very significant (**) and less than 0.01% statistically extremely significant (***).

RESULTS

LIGAND-DEPENDENT ACTIVATION OF THE ER-α TRANSCRIPTION FACTOR IN HUMAN MCF-7 BREAST CANCER CELLS

Initially, immunoblotting was used to determine the basal and inducible levels of expression and the functional status of ER-α in the tested human breast cancer cells. As expected, human MCF-7 breast cancer cells (the mother cell line, cells expressing HPV-encoded E6 oncoprotein, and cells transfected with an empty CMV vector) expressed ER-α, but 2 of over 10 breast cancer cell clones examined were ER-α-negative. In Figure 1A WCLs prepared from human HeLa cervix carcinoma cells and four breast cancer cell clones were analyzed. Remarkably, BT-20 were ER-α-negative and in cells expressing HPV-encoded E6 oncoprotein ER-α level was slightly reduced (Fig. 1A, 4th lane). In contrast, DBC1 was present in whole-cell lysate (WCL) samples from all of the tested cell lines. Human MCF-7 breast cancer cells in exponential growth phase were

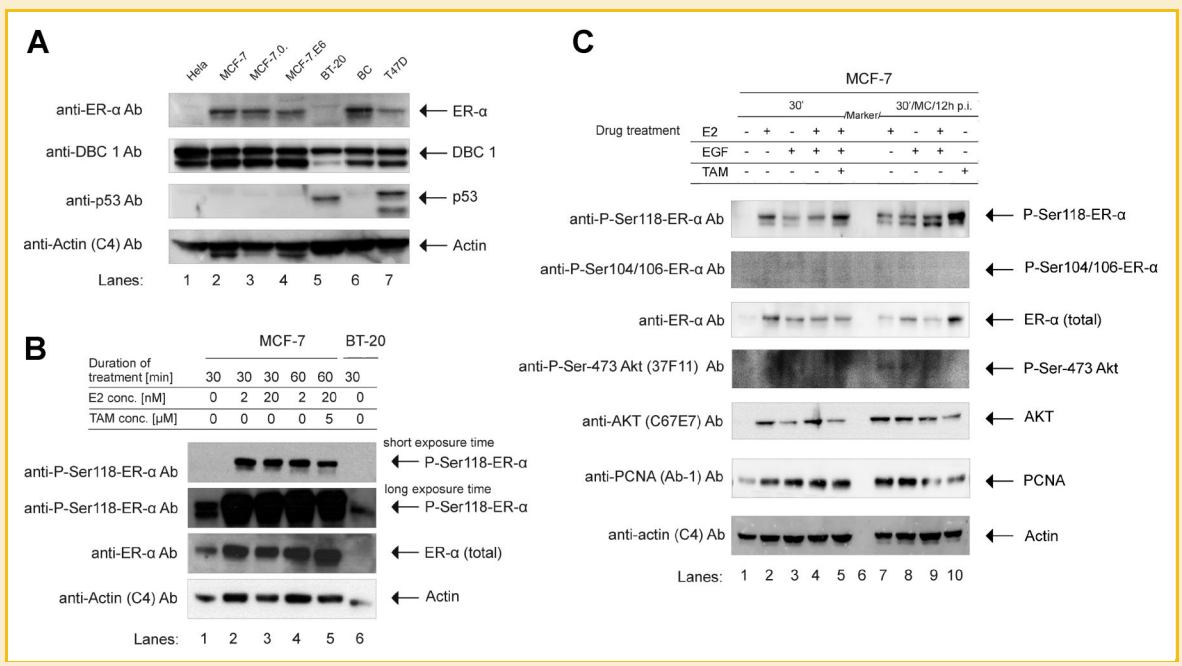

Fig. 1. Ligand-dependent activation of the ER-α transcription factor in human MCF-7 breast cancer cells. A: Selection of ER-α-positive and -negative cells from a collection of established breast cancer cells lines. WCLs prepared from control human HeLa cervix carcinoma cells and four breast cancer cell lines were analyzed by immunoblotting after separation on 10% SDS gels. Blots were incubated with antibodies directed against ER-α, DBC 1, and p53 protein. Immune complexes were detected after incubation with secondary antibodies linked to HRP and chemiluminescence reagent ECL Plus™ (GE Healthcare, formerly Amersham Biosciences). Chemiluminescence was monitored using ChemiSmart5100 apparatus (PEQLAB, Biotechnologie GmbH, Erlangen, Germany). B: Determination of a basal and estrogen-inducible phosphorylation of ER-α at Ser^{118}. Human MCF-7 cells collected prior to the onset of treatment and after treatment with estrogen were lysed and analyzed by immunoblotting as described in detail in (A). Chemiluminescence was detected after exposing the blots to film. To visualize the basal phosphorylation status of ER-α at Ser^{118}, blots were long exposed to film. C: Estimation of duration of ligand-dependent activation ER-α. Human MCF-7 cells collected directly after treatment with drugs as indicated or after post-incubation in a drug-free medium for 12 h were lysed and analyzed by immunoblotting as described in detail in (A). Phosphorylation of ER-α at $Ser^{104/106}$ and at Ser^{118} as well as phosphorylation of AKT at Ser^{473} was determined.

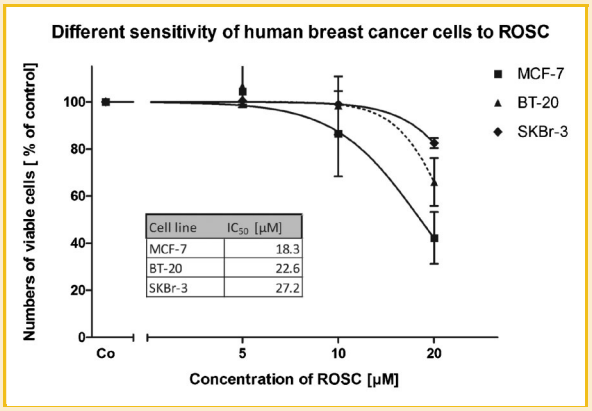

Fig. 2. Different sensitivity of human breast cancer cells to treatment with ROSC. Exponentially growing estrogen-responsive MCF-7 cells and two ER-α-negative cell lines (BT-20 and SKBr-3) were plated in 96-well microtiter plates and 24 h after plating were treated with ROSC at indicated concentrations for 24 h. The numbers of viable cells were determined directly after the treatment using CellTiter-Glo™ assays (Promega Corporation, Madison, WI). The data represent mean values from three independent experiments, each performed at least in quadruplicate. Results were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Dose–response curves were calculated by nonlinear regression analyses. IC_{50} values were determined from dose–response curves.

then exposed to estradiol (E2) for 30 or 60 min at final concentrations of 2 or 20 nM with or without TAM. The untreated control cells and the cells treated with drugs were harvested and lysed, and their WCLs were analyzed by immunoblotting. The blots were first probed with antibodies directed solely against ER-α phosphorylated at Ser^{118} and then with antibodies recognizing ER-α without regard to post-translational modifications. As shown in Figure 1B, phosphorylated ER-α was barely detectable in samples from the untreated MCF-7 control cells (1st lane). Indeed, the basal level of ER-α phosphorylation could only be detected by prolonged exposure of the immunoblot (Fig. 1B). E2 is the natural ligand of ER-α; in its presence, extensive phosphorylation of Ser^{118} was observed, accompanied by an increase in the expression of the receptor. A relatively low dose of the hormone (2 nM) was sufficient to induce strong activation of ER-α, and treatment with a higher concentration of E2 (20 nM) did not further increase the extent of phosphorylation of ER-α at Ser^{118}. Furthermore, we assessed the ability of E2 and/or EGF to induce ER-α phosphorylation at two adjacent serine residues (e.g., Ser^{104}/Ser^{106}) that are known to be modified under certain conditions (Fig. 1C). Within 30 min of treatment of MCF-7 cells with E2 or EGF, the hormone receptor became phosphorylated at Ser^{118} but not at Ser^{104}/Ser^{106} (Fig. 1C). Co-treatment with TAM slightly enhanced the phosphorylation of ER-α at Ser^{118}. This was particularly evident after 12 h post-incubation in a drug-free medium and was associated with an increase in the total level of the nuclear receptor (Fig. 1C, 10th lane). A WCL generated from control BT-20 cells was used as a negative control (Fig. 1B, 6th lane). However, treatment of MCF-7 cells with E2 or EGF induced a weak phosphorylation of AKT at Ser^{473} (Fig. 1C, 7th lane) and increased the total level of the kinase. This confirms our previous results [Wesierska-Gadek et al., 2011a] and indicates that ligand-mediated stimulation ER-α was specific. The results demonstrate that ER-α is functional in MCF-7 cells and is strongly activated even by brief exposure to E2.

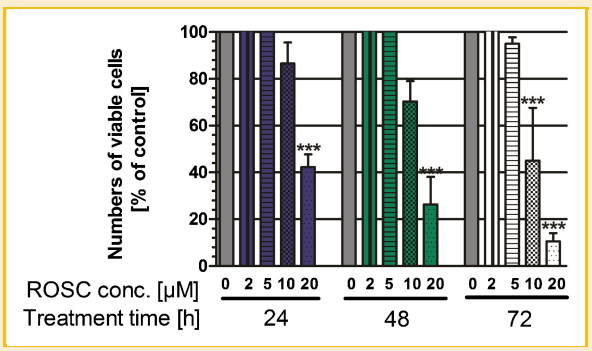

Fig. 3. Long exposure of MCF-7 cells to ROSC potentiates its anti-proliferative effect. Exponentially growing MCF-7 cells plated in 96-well microtiter plates were treated for 24, 48, and 72 h with indicated concentrations of ROSC. The numbers of viable cells were determined directly after the continuous treatment using CellTiter-Glo™ assays (Promega Corporation, Madison, WI). The data represent mean values from three independent experiments, each performed at least in quadruplicate. Results were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Statistical significance of the reduction of cell numbers after treatment was calculated using Bonferroni's multiple comparison test. The effect of 20 μM ROSC was statistically extremely high significant at all treatment points ($P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ROSC HAS A STRONGER EFFECT ON PROLIFERATION IN ER-α-POSITIVE HUMAN BREAST CANCER CELLS

Three cell lines were used to determine the anti-proliferative effects of ROSC on human breast cancer cells with differing levels of expression of ER-α: ER-α-positive MCF-7 cells, and two cell lines (BT-20 and SKBr-3) lacking ER-α. Cells were plated in microtiter plates and exposed to a range of concentrations of ROSC for 24 h. The numbers of living cells were then determined using the CellTiter-Glo™ assay. The drug decreased the number of viable breast cancer cells in a concentration-dependent manner. Remarkably, ROSC treatment had a much stronger effect on the proliferation rate of ER-α-positive MCF-7 cells ($IC_{50} = 18.3$ μM) than on ER-α-negative BT-20 ($IC_{50} = 22.6$ μM) and SKBr-3 cells (27.2 μM; Fig. 2).

LONG EXPOSURE OF MCF-7 CELLS TO ROSC POTENTIATES ITS ANTI-PROLIFERATIVE EFFECT

Of the three human breast cancer cell lines examined, MCF-7 cells displayed the highest sensitivity to the inhibition of cellular CDKs after 24 h. To determine whether longer continuous treatment would potentiate the anti-proliferative effect of the CDK inhibitor, a second series of experiments in which the duration of the ROSC treatment was extended to 48 or 72 h was conducted. Such extended treatment markedly enhanced the anti-proliferative effect of the CDK inhibitor (Fig. 3). The number of living MCF-7 cells was halved after exposure to 10 μM ROSC for 72 h; this reduction in cell numbers was highly significant. Moreover, ROSC-mediated inhibition of proliferation (24 h) was maintained in MCF-7 cells even after wash-out and post-incubation of cells for a further 24 h in a drug-free medium (not shown).

ROSC ARRESTS THE CELL CYCLE PROGRESSION OF ASYNCHRONOUSLY GROWING MCF-7 CELLS

To determine how ROSC modulates the cell cycle progression of human MCF-7 breast cancer cells, exponentially growing cells were exposed to ROSC for 24 h at final concentrations of 10, 20, and 40 μM. Cells were then harvested and the DNA concentration of single cells was determined by flow cytometric measurement of their fluorescence intensity following propidium iodide staining. As expected, treatment of MCF-7 cells with ROSC for 24 h induced arrest at the G_2/M transition (Fig. 4). The proportion of G_2-phase

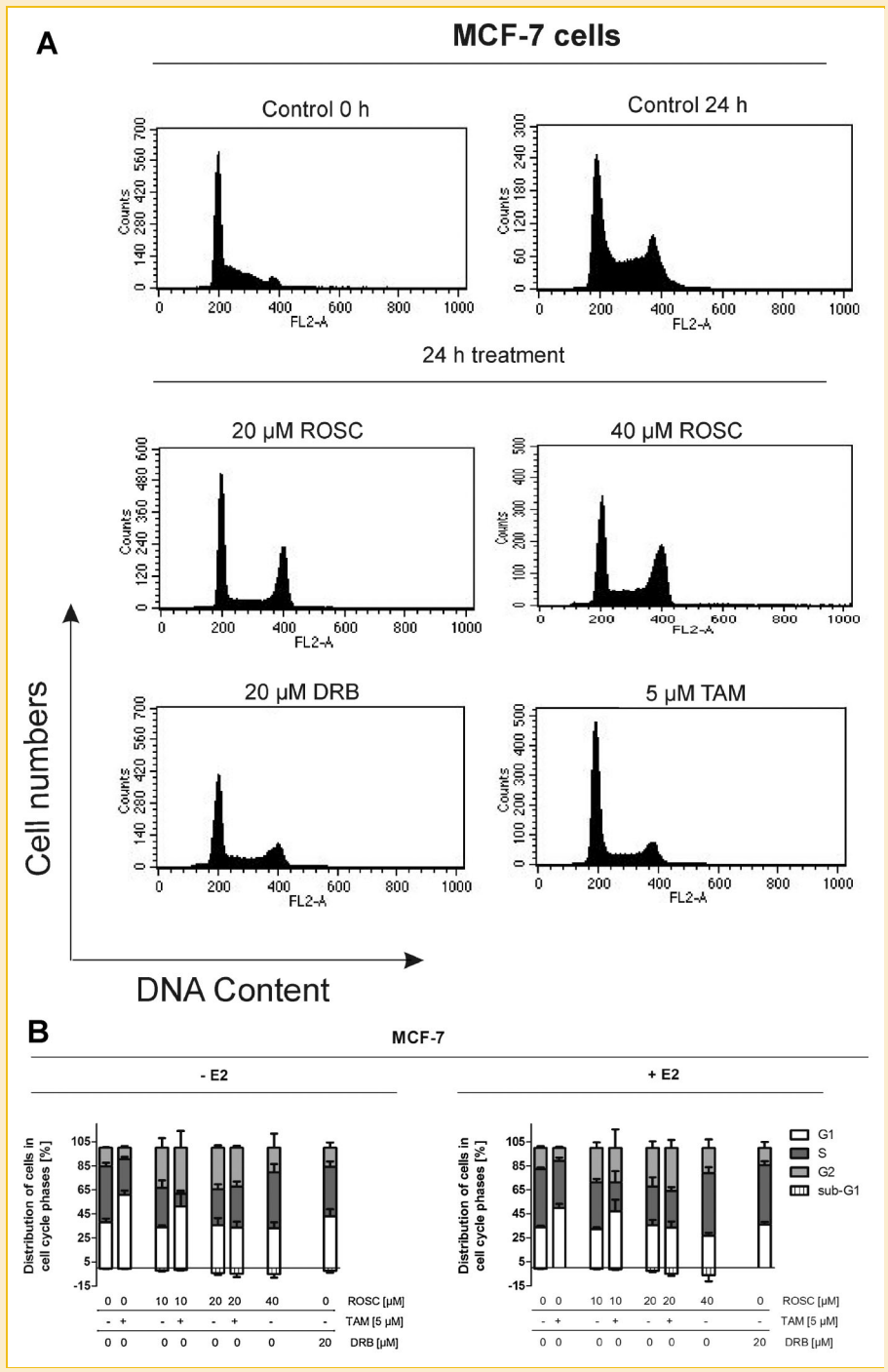

Fig. 4. ROSC but not DRB induces accumulation of G_2 arrested MCF-7 cells. Exponentially growing human MCF-7 cells were treated with ROSC ($C_E = 10, 20, 40$ μM), DRB ($C_E = 20$ μM), or TAM ($C_E = 5$ μM) for 24 h. Cells were harvested and stained with propidium iodide. DNA content in single cells was measured by flow cytometry. A: DNA profiles depicting the effect of CDK inhibitors after treatment for 24 h. DNA histograms obtained from a representative experiment performed in duplicate were prepared using Cell Quest software. B: Comparison of the CDK inhibitors on the distribution of cells in specific cell cycle phases. DNA concentrations were evaluated using ModFIT software. The data represent mean values ± SD from three independent experiments.

cells increased twofold from 15% to 33% after treatment with 10 μM ROSC (or to 34% with 20 μM ROSC) as compared with the untreated control cells. In cells treated with 10 μM ROSC, G_2 arrest was accompanied by a reduction in the proportion of S-phase cells. Remarkably, treatment with 40 μM ROSC caused an increase in the proportion of S-phase cells (Fig. 4). These changes in the distribution of cells in different phases of the cell cycle seem to be attributable to the inhibition of CDK2 by ROSC because DRB, a known inhibitor of cellular kinases involved in RNA synthesis [te Poele et al., 1999] did not affect cell cycle progression (Fig. 4). ROSC also induced accumulation of hypoploid cells, that is, cells undergoing apoptosis. After treatment for 24 h with 20 μM ROSC the ratio of sub-G_1 cells increased from 0.6% in the untreated controls to 4%.

AT HIGHER DOSES, ROSC DISRUPTS THE MITOCHONDRIAL MEMBRANE POTENTIAL

DNA analyses indicated the presence of sub-G_1 cells in MCF-7 samples treated with higher doses of ROSC. MCF-7 cells do not express caspase-3 and therefore are relatively resistant to apoptosis-inducing agents. To assess the effect of ROSC treatment on the integrity of the mitochondrial membrane, the membrane potential was monitored using the electrochromic dye JC-1 [Reers et al., 1991]. JC-1 is a carbocyanine bearing a delocalized-positive charge, and its distribution between mitochondrial compartments is dependent on the membrane potential. The membrane potential of energized mitochondria (which have a lower potential inside the matrix than outside) promotes uptake of JC-1 into the matrix and subsequent formation of J-aggregates. Untreated controls and cells treated with ROSC or STAU (a strongly cytotoxic kinase inhibitor) at the indicated concentrations for 24 h were detached from the substratum by limited proteolysis and then incubated with JC-1 dye. After thorough washing, the cells were immediately analyzed by flow cytometry. J-aggregates were observed in almost all of the untreated control cells (95% of gated cells). However, such aggregates were not observed in a number of MCF-7 cells exposed to ROSC or to STAU for 24 h; instead green fluorescence attributable to the monomeric dye was seen (Fig. 5). Bivariate flow cytometric analyses revealed 3- and 7-fold increases in the numbers of cells emitting green fluorescent signals after treatment with 20 and 40 μM ROSC, respectively. Treatment of cells with STAU caused a 13-fold increase in the proportion of green fluorescent cells (Fig. 5).

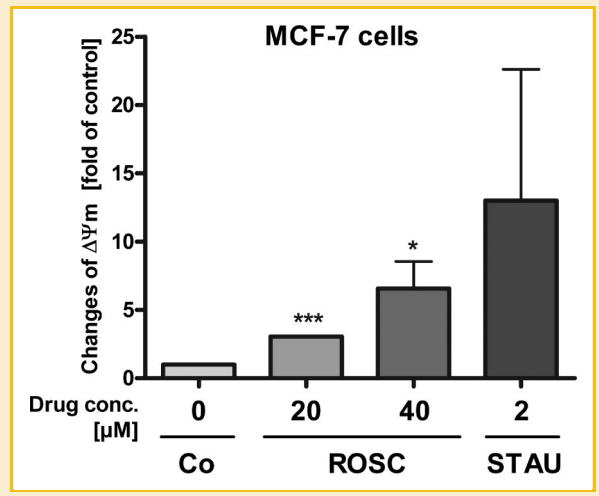

Fig. 5. At higher doses, ROSC disrupts the mitochondrial membrane potential. Untreated MCF-7 control cells and cells treated with ROSC at indicated concentrations for 24 h were collected and incubated with JC-1 dye as described in detail in the Experimental Section. Directly after washing cells were subjected to two-color analysis by flow cytometry. The diagram shows the increase of the population of cells that lost the capability to aggregate JC-1 dye as compared to untreated controls. The data represent mean values ± SD from two independent experiments. The statistical analyses were performed using SPSS Statistic Base 17.0 software (SPSS Inc., Chicago, Il). Prior to the statistical analysis of the data, the ratios of cells not capable to aggregate JC-1 dye were logarithmically transformed. The analysis was performed using t-test. The reported *P*-value is a result of a two-sided test. A *P*-value 5% to 1% is considered statistically significant (*), 1% to 0.1% statistically very significant (**) and less than 0.01% statistically extremely significant (***). STAU is strongly cytotoxic, and MCF-7 cells treated for 24 h with 2 μM STAU were used to ensure that test properly works.

SERMs INDUCE G_1 ARREST AND SLOWER PROLIFERATION OF MCF-7 CELLS

Selective estrogen response modifiers, such as TAM or its active metabolite (4-OHT), are routinely used in the treatment of ER-α-positive breast cancers in combination with other drugs. Exposure of exponentially growing MCF-7 cells to both of these SERMs reduced the numbers of living cells in a time- and concentration-dependent manner. Remarkably, 4-OHT, an active TAM metabolite, inhibited proliferation of MCF-7 at several concentrations within 24 h of treatment, whereas the effects of TAM on MCF-7 cells only became apparent after 48 h exposure (Fig. 6). In contrast, the SERMs had much weaker effects on BT-20 and SKBr-3 cells (data not shown). DNA content analysis using flow cytometry revealed that TAM reduced the proportion of MCF-7 cells in S-phase by approximately 30%, but had a less pronounced effect on SKBr-3 cells (not shown). This decrease in the population of S-phase cells was accompanied by an accumulation of cells in the G_1 phase as compared to the control MCF-7 cells that had been incubated for 24 h without exposure to drugs (Fig. 4).

INTERFERENCE WITH ER-α BY TAM ENHANCES THE ANTI-PROLIFERATIVE ACTION OF ROSC, PARTICULARLY IN ER-α-POSITIVE BREAST CANCER CELLS

We next sought to determine whether TAM might modulate the efficacy of pharmacologically relevant CDK inhibitors. Comparatively low concentrations of the CDK inhibitor and the SERM were used in these combination experiments, in which MCF-7 cells were exposed for 24 and 48 h to a range of concentrations of ROSC, both alone and in combination with 5 μM TAM. The number of viable cells was then determined using the CellTiter-Glo™ assay. TAM did not sensitize MCF-7 cells to ROSC after treatment for 24 h (Fig. 7A). TAM did not decrease the ROSC concentration required for a 50% reduction in the number of viable cells (Fig. 7A). IC_{50} values for ROSC alone and in combination with 5 μM TAM increased by approximately 25% after co-treatment with E2 (Fig. 7A). However, compared to treatment with ROSC alone for 48 h, the combination of ROSC with 5 μM TAM had significantly greater inhibitory effects on

TABLE I. Synergistic Cooperation Between ROSC and TAM in MCF-7 Cells

	5 μM TAM			
	5 μM ROSC	10 μM ROSC	20 μM ROSC	40 μM ROSC
24 h				
Without E_2	1.176	1.621	1.004	1.191
With E_2	0.574	0.699	1.088	1.687
48h				
Without E_2	0.874	0.826	0.843	1.089
With E_2	0.482	0.460	0.526	0.728

Comparison of the combination index (CI) calculated for each ROSC concentration.
Naive and E2-stimulated MCF-7 cells were treated for 24 and 48 h with 5 μM TAM combined with ROSC at indicated concentrations. Combination index (CI) was calculated using CalcuSyn software (Version 2).

MCF-7 cells and reduced the ROSC concentration required for a 50% reduction in the number of viable cells by a factor of almost 2.5 (Fig. 7B). The interaction between the two compounds was analyzed using CalcuSyn software. The calculated CI was <1 for all three ROSC concentrations tested, demonstrating the synergistic action of 5 μM TAM with ROSC at concentrations ranging from 5 to 20 μM (Table I). The interaction was additive (CI = 1.089) when 40 μM ROSC was combined with 5 μM TAM. Remarkably, co-treatment with E2 enhanced the synergistic action of 5 μM TAM with ROSC and reduced the ROSC concentration required for a 50% reduction in the number of viable cells by a factor of almost 4. Flow cytometric DNA content analysis revealed that the combination of TAM with lower ROSC doses decreases the population of S-phase cells (data not shown). How to explain the potentiation of anti-proliferative action of ROSC by TAM arising after treatment for 48 h? The impact of anti-estrogens (TAM and 4-OHT) at low concentrations becomes evident after treatment for at least 48 h. Therefore, it is not surprising that cooperative action of 5 μM TAM with ROSC not developed until 48 h.

Interestingly, a weak enhancement of the anti-proliferative effect of ROSC by TAM was also observed in the two cell lines lacking ER-α: SKBr-3 and (to a lesser extent) BT-20 (data not shown). For these cases, the CIs calculated by interaction analysis were >1, indicating a lack of synergy between TAM and ROSC in ER-α-negative breast cancer cells.

ACTIVATION OF ER-α IN MCF-7 CELLS ENHANCES THE EFFICACY OF THE ROSC + TAM COMBINATION

As shown in Figure 1, the basal level of ER-α phosphorylation in MCF-7 cells cultivated in a phenol-free medium is very low, but it rises dramatically after brief exposure to its natural ligand. Therefore, our final objective was to determine whether the activation of ER-α would augment the synergy between TAM and ROSC. This proved to be the case: the anti-proliferative activity of ROSC was greatly enhanced in MCF-7 cells with activated ER-α (Fig. 7). The IC_{50} value of ROSC in these cells was decreased by a factor of approximately 4 in the presence of TAM. Remarkably, synergistic interactions between ROSC and TAM were observed at all ROSC concentrations examined in cells with activated ER-α (Table I).

ROSC CHANGES THE FUNCTIONAL STATUS OF CELL CYCLE REGULATORS

The primary target of ROSC is CDK2. To determine the dose required to inhibit this kinase, exponentially growing MCF-7 cells treated with different concentrations of ROSC were collected at 8-h intervals following the onset of treatment (8, 16, and 24 h), lysed and analyzed by immunoblotting. A slight decrease in the extent of CDK2 phosphorylation at Thr^{160} was observed after 16 h exposure to ROSC (Fig. 8). After 24 h treatment with ROSC at a final concentration of 40 μM, phosphorylation of CDK2 and CDK7 was almost unobservable (Fig. 8). These results clearly demonstrate that ROSC becomes fully operative in MCF-7 cells after 24 h exposure.

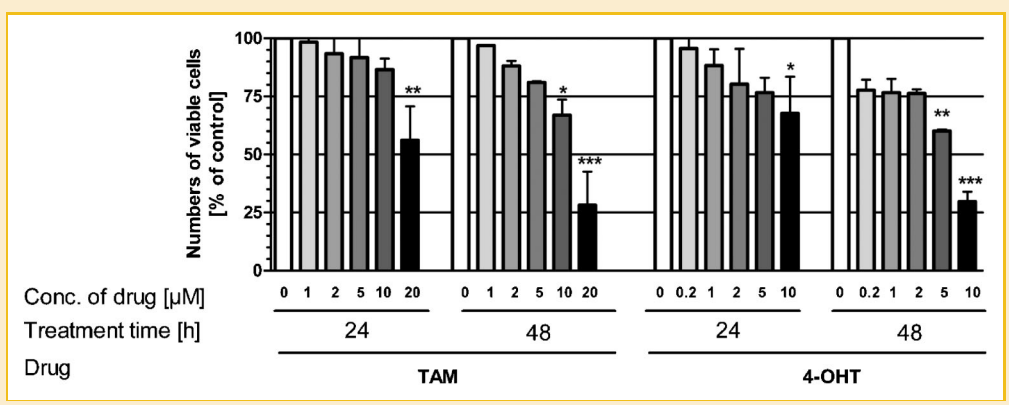

Fig. 6. SERMs slow proliferation of MCF-7 cells. Exponentially growing MCF-7 cells plated in 96-well microtiter plates were treated for 24 and 48 h with TAM or 4-OHT at indicated concentrations. The numbers of viable cells were determined directly after the continuous treatment using CellTiter-Glo™ assays (Promega Corporation, Madison, WI). The data represent mean values from three independent experiments, each performed at least in quadruplicate. Results were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Statistical significance of the reduction of cell numbers after treatment was calculated using Bonferroni's multiple comparison test. The effect of 20 μM TAM was statistically extremely significant after treatment for 48 h ($P<0.001$).

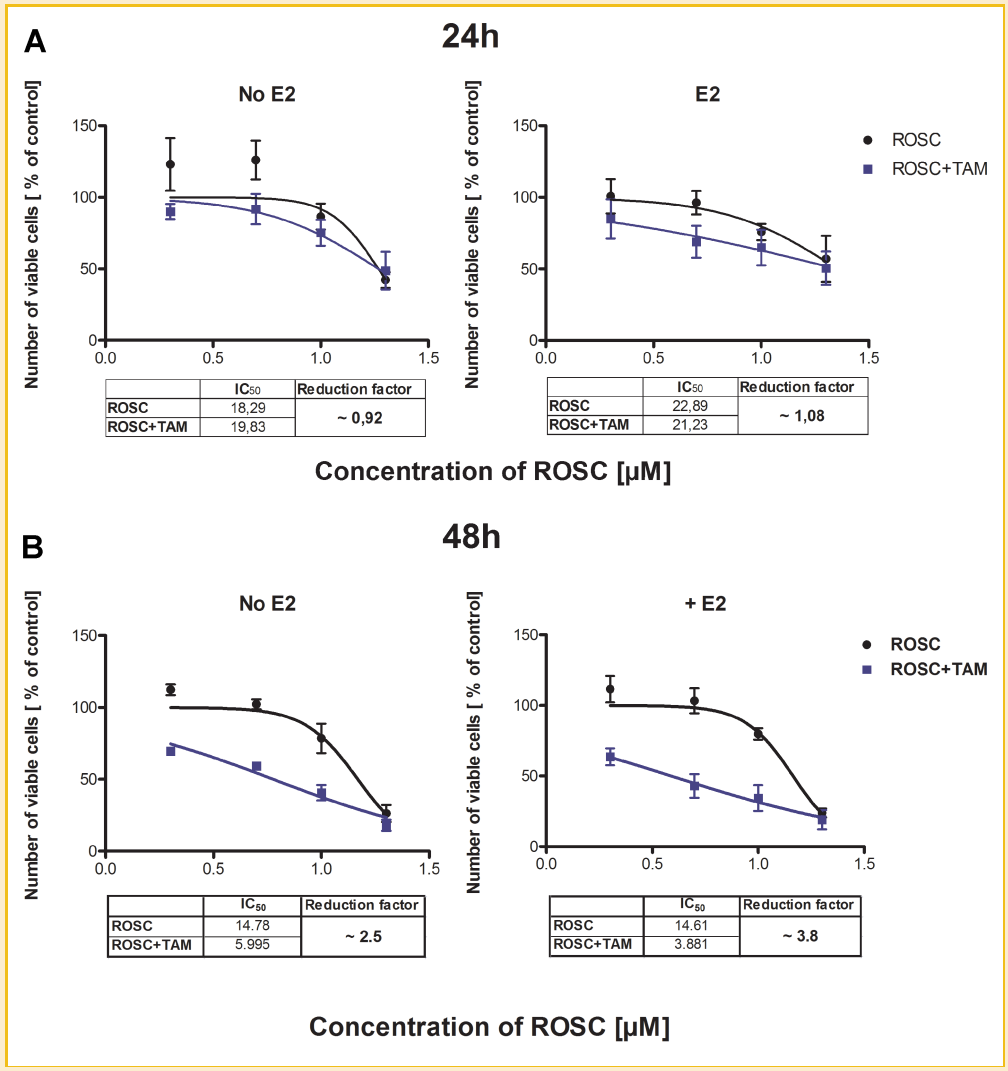

Fig. 7. Interference with ER-α by TAM enhances the anti-proliferative action of ROSC, particularly in ligand-stimulated ER-α-positive breast cancer cells. Exponentially growing MCF-7 cells were plated in 96-well microtiter plates. Twenty-four hours after plating cells were exposed to ROSC alone or ROSC combined with TAM for 24 h (A) or for 48 h (B) in the presence or absence of E2. After termination of continuous treatment, the numbers of living cells were determined using the CellTiter-Glo™ luminescent cell viability assay (Promega Corporation, Madison, WI). The data represent mean values from three independent experiments, each performed at least in quadruplicate. ROSC concentration is shown in logarithmic scale. Results were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). The effects of the combined ROSC and TAM treatments on the IC_{50} values shown are defined as reduction factors (reduction factor = IC_{50} ROSC/IC_{50} ROSC + TAM). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ROSC ABOLISHES BASAL PHOSPHORYLATION OF ER-α

The extent of the activating phosphorylation of CDK7 in MCF-7 cells exposed to ROSC for 24 h was greatly reduced (Fig. 8). Therefore, we assessed the impact of this inactivation of CDK7 on the phosphorylation of ER-α. As previously shown in non-stimulated MCF-7 cells, ER-α may be phosphorylated at Ser^{118}, but not at $Ser^{104/106}$ [Gritsch et al. 2011; Wesierska-Gadek et al., 2011a; Zulehner et al., 2011]. Exposure of MCF-7 cells to ROSC caused a decrease in the extent of phosphorylation of ER-α at Ser^{118} in a concentration-dependent manner (approximately 100% at 40 μM; Fig. 9, 2nd lane). The total cellular concentration of ER-α decreased by 80% after treatment with 40 μM ROSC (Fig. 9, 2nd lane). In the latter case, the reduction in ER-α phosphorylation coincided with a strong reduction in the phosphorylation of Ser^{5} of the carboxyl-terminal repeat domain (CTD) of RNA Pol II, indicating the presence of a global block on transcription. DRB, an inhibitor of CDK9, reduced neither the phosphorylation of ER-α nor its total cellular levels (Fig. 9, 3rd lane). Notably, ROSC strongly induced the p53 tumor suppressor protein, resulting in its accumulation in MCF-7 cells (Fig. 9) and in nuclei due to phosphorylation of Ser^{46} (not shown). Furthermore, ROSC decreased phosphorylation of survivin, an inhibitor of apoptosis, at Thr^{34}, which destabilizes the protein (not shown). The phosphorylation of this residue is catalyzed by activated CDK1; when so modified, survivin becomes stable and prevents apoptosis during mitosis. The decrease in cellular survivin levels correlated with an increase in the proportion of cells having a mitochondrial membrane potential (Fig. 5). Finally, both 4-OHT and TAM caused an increase (Fig. 9, lanes 4–5) in the total level of the

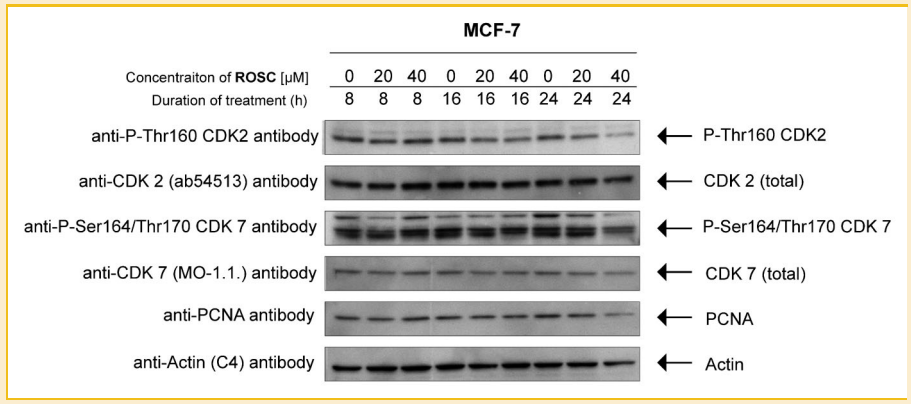

Fig. 8. ROSC alters the functional status of cell cycle regulators. MCF-7 cells treated with different concentrations of ROSC were collected at 8-h intervals following the onset of treatment (8, 16, and 24 h), lysed and analyzed by immunoblotting. WCLs prepared from untreated control cells and cells exposed to ROSC were separated on a 12% SDS gel (30 μg/lane) and transferred onto a PVDF membrane. The blots were incubated with indicated primary antibodies recognizing antigen phosphorylated at defined position in an appropriate concentration, and sequentially with antibodies directed against total protein. Equal protein loading was confirmed by Ponceau S staining of the membrane and by incubation with anti-actin.

ER-α hormone receptor. However, the extent of its phosphorylation markedly increased (Fig. 9, 4th and 5th lane).

ROSC INACTIVATES ER-α IN LIGAND-STIMULATED MCF-7 CELLS

Finally, we sought to determine whether ROSC can inhibit CDK7 and consequently abolish site-specific phosphorylation of the steroid receptor in MCF-7 cells that have been exposed to estrogen and to EGF. Untreated breast cancer cells and cells treated with 40 μM ROSC, 20 μM DRB, 5 μM TAM, or 0.5 μM 4-OHT for 24 h were exposed for 30 min to the hormone. MCF-7 cells stimulated with estrogen exhibited increased levels of activating phosphorylation of CDK2 and CDK7 (Fig. 9, 5th lane), which persisted for 24 h (not shown). In contrast, cells treated solely with TAM exhibited markedly reduced phosphorylation of the CDKs and the pRb

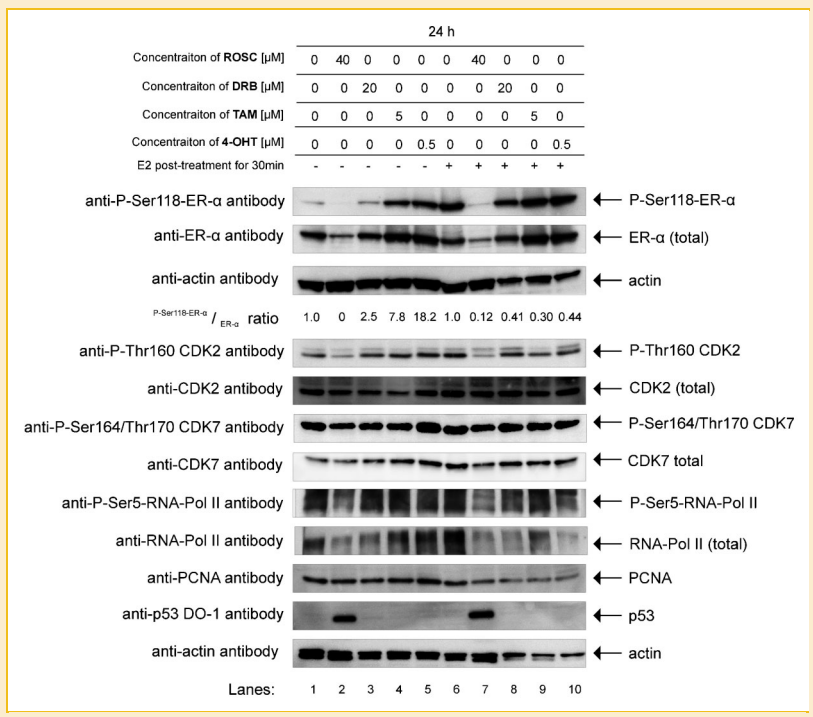

Fig. 9. ROSC abolishes basal and ligand-induced phosphorylation of ER-α. WCLs prepared from control MCF-7 cells and cells treated with ROSC, DRB, TAM or 4-OHT were separated on 10% or 8% SDS gels and transferred onto the PVDF membranes. Blots were incubated with primary antibodies as indicated. Immune complexes were detected after incubation with secondary antibodies linked to HRP and chemiluminescence reagent ECL+. Chemiluminescence was monitored using ChemiSmart5100 apparatus (PEQLAB, Biotechnologie GmbH, Erlangen, Germany). The intensity of protein bands representing P-Ser^{118} ER-α and total ER-α protein in each lane was normalized against actin. Then P-Ser^{118} ER-α/ER-α ratio was calculated and normalized against the ratio calculated for the control sample (100).

protein, but also strongly increased levels of the ER-α receptor. Simultaneous treatment with E2, EGF, and TAM did not counteract either the activation of CDKs or that of ER-α observed after stimulation (Fig. 1C). In contrast, the presence of ROSC during the 24 h post-incubation period (not shown) resulted in decreased phosphorylation of the CDKs, pRb protein and ER-α, and reduction of protein concentrations. The changes were even more pronounced than in naïve cells treated with ROSC (not shown). These results demonstrate that ROSC abolishes both basal and ligand-induced phosphorylation of ER-α at Ser^{118} and confirm our previously reported observations [Wesierska-Gadek el al., 2011a].

DISCUSSION

ER-α is the most clinically important biomarker in oncology. Approximately 60% of breast cancers are ER-α-positive and lack amplification of the *HER2* gene [Hulka and Stark, 1995; Glass et al., 2007]. Such $ER^+/HER2^-$ breast cancers typically respond well to targeted endocrine therapy, which suppresses estrogen signaling in malignant cells [EBCTCG, 2005; Glass et al., 2007]. Unfortunately, however, breast cancers acquire resistance during the course of endocrine therapy [EBCTCG, 2005]. Therefore, new therapeutic options must be identified.

The deregulation of cell cycle control is one of the hallmarks of cancer; it gives malignant cells unlimited replicative potential [Hanahan and Weinberg, 2000] and seems to influence survival in high-risk breast cancer patients [Konigsberg et al., 2008]. In general, two of the most frequently observed molecular events associated with cancer are enhanced activity of CDK/cyclin complexes and the inactivation and/or loss of cellular inhibitors of CDKs [Malumbres and Barbacid, 2009]. Some of the defects specifically associated with breast cancer include: increased levels of cyclins D_1 [Bartkova et al., 1994] and E [Keyomarsi et al., 1995; Spruck et al., 1999], which promote the activation of kinase complexes regulating progression through the G_1 phase and the G_1/S transition [Harwell et al., 2004]; inactivation of the cellular *INK4A* gene, which encodes both the $p16^{INK4A}$ protein (a cellular CDK inhibitor) and the $p14^{ARF}$ protein (which regulates the interaction between Mdm2 and the wt p53 tumor suppressor protein); and decreased levels of the $p27^{Kip1}$ protein [Sherr and Roberts, 1999; Senderowicz, 2003]. The deregulation of cyclin E induces chromosomal instability [Spruck et al., 1999]. Therefore, the use of pharmacological inhibitors that affect key cellular CDKs to target-specific components of the cell cycle constitutes a promising therapeutic strategy. Over the last 20 years, several CDK inhibitors have been developed; of these, approximately 20 compounds have proceeded to clinical trials or beyond [Węsierska-Gądek et al., 2009b].

ROSC, a 2,6,9-trisubstituted purine analogue (also known as Selicliclib™; CYC-202; Cyclacel Ltd.) competes for binding to the ATP pocket of the kinase catalytic subunit [De Azevedo et al., 1997; Havlicek et al., 1997; Benson et al., 2007]. Although it affects several kinases, it primarily inhibits CDK2 and CDK7 [McClue et al., 2002; Wesierska-Gadek et al., 2009a]. The inhibition of CDK2, a master regulator of the G_1/S phase transition, results in cell cycle arrest in cells originating from breast cancer [David-Pfeuty, 1999; Wojciechowski et al., 2003; Wesierska-Gadek and Schmid, 2006] and other aggressive tumors [Wesierska-Gadek et al., 2009a; Galimberti et al., 2010]. However, the biological effects of ROSC are strongly dependent on its dose, the duration of treatment, cell type, and cellular context. ROSC is known to activate wild-type (wt) p53 [David-Pfeuty, 1999; Kotala et al., 2001; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Therefore, treatment of cancer cells harboring a functional p53 tumor suppressor gene benefits from this additional aspect of ROSC activity [Wesierska-Gadek et al., 2009a]. Moreover, ROSC inhibits the specific phosphorylation of CDK7. CDK7 phosphorylates RNA polymerase II (RNA Pol II) and thereby renders it competent in transcriptional elongation. Thus, the inhibition of CDK7 phosphorylation by ROSC results in a global transcriptional block [Bres et al., 2008; Core and Lis, 2008]. Abolishing the specific phosphorylation of CDK7 and RNA Pol II reduces cellular levels of important inhibitors of apoptosis and thus promotes programmed cell death in tumor cells [Hahntow et al., 2004; Alvi et al., 2005; Wesierska-Gadek et al., 2009a; Rogalinska et al., 2010]. These varied facets of responses to ROSC indicate that it has great potential in the clinical treatment of ER^+ breast cancer.

It is well documented that estrogen-mediated pathways play a central role in the proliferation and survival of breast cancer cells [Cheskis et al., 2007]. To date, two ERs (ER-α and ER-β) encoded by two separate genes have been identified; of these, ER-α is a very potent transcription factor [Aronica and Katzenellenbogen, 1993; Kato et al., 1995; Katzenellenbogen et al., 1996; Brzozowski et al., 1997] and a key integrator of estrogen signaling [Le Goff et al., 1994; Joel et al., 1995, 1998; Chen et al., 2000]. By analyzing expression profiles, 61 intrinsically estrogen-regulated genes that serve as specific markers of ER-α-positive breast cancer have been identified [Weisz et al., 2004]. The expression, functional status, and activity of ER-α are extremely complex. In addition to its full-length form, shorter variants are generated by alternative splicing and may modulate the receptor's transcriptional activity. Its regulation and behavior are further complicated by variations in its localizations and modes of transactivation (classical and non-classical), the recruitment of several co-regulator molecules (co-activators and co-repressors), and the organization of its ligand-binding domain (LBD), which harbors additional elements that modulate its activity [reviewed in Cheskis et al., 2007].

In this study, we explored the consequences of targeting CDK2, CDK7, and ER-α in human breast cancer cells. ROSC was found to efficiently inhibit the proliferation of three breast cancer cell lines, of which the ER-α-positive MCF-7 line was most strongly affected. Detailed analyses revealed that ROSC affects the functional status of a few key molecules. As expected, it abolishes the activating phosphorylation of CDK2 at Thr^{160}. Phosphorylation of CDK2 at this specific residue within the T-loop changes its conformation from "closed" to "opened," enabling substrate binding [Węsierska-Gądek et al., 2009b]. Furthermore, the extent of phosphorylation of survivin at Thr^{34}, which is catalyzed by the activated CDK1/cyclin B complex, was reduced in ROSC-treated MCF-7 cells, indicating that both CDK1 and survivin had been deactivated. Dephosphorylation of survivin, a small inhibitor of apoptosis, renders it unstable and susceptible to degradation. ROSC also caused a decrease in the phosphorylation of CDK7, and, consequently, in the phosphoryla-

tion of Ser^{5} in the COOH-terminal domain (CTD) of RNA Pol II. The suppression of this last phosphorylation is specific to ROSC: treatment with DRB, an inhibitor of CDK9, did not affect the phosphorylation of this residue. We then examined the influence of CDK7 inactivation on the functional status of ER-α. ROSC abolished phosphorylation of ER-α at Ser^{118} [Wesierska-Gadek et al., 2011a], the residue modified in response to estrogen signaling [Joel et al., 1995]. Remarkably, ROSC also effected a reduction in the total level of the receptor. However, inhibition of transcriptional elongation by DRB affected neither the phosphorylation of ER-α at Ser^{118} nor its cellular levels, indicating that the observed changes in ER-α are attributable to the inhibition of CDK7. Remarkably, ROSC strongly activated the p53 tumor suppressor protein in MCF-7 cells by phosphorylation at Ser^{46}, resulting in its accumulation in the nuclei. Again, DRB failed to upregulate cellular levels of p53, thus demonstrating that a block of global transcription is not sufficient to enhance p53 levels in MCF-7 cells. Significantly, ROSC abolished both basal and ligand-induced phosphorylation of ER-α at Ser^{118}. These results explain the mechanisms by which ROSC reduces the proliferation of naive and estrogen-stimulated MCF-7 cells. We also investigated the influence of SERMs on the activity of ROSC. TAM clearly enhanced the anti-proliferative effect of ROSC; this interaction was synergistic at all concentrations of the CDK inhibitor in ligand-stimulated MCF-7 breast cancer cells after treatment for 48 h. Our results indicate that the mechanism by which the ROSC-TAM combination effects inhibition is similar to that of ROSC alone.

The strong activation of p53 observed following treatment with ROSC suggests that p53 activation might affect the efficacy of a combined ROSC-SERM therapy. The activation of wt p53 in ROSC-treated MCF-7 cells seems to be important for the induction of the mitochondrial apoptosis pathway [Wesierska-Gadek et al., 2005a]. We have previously reported that ROSC induces phosphorylation of p53 at Ser^{46} and that this modification precedes both an increase of p53 levels and the induction of apoptosis by disruption of the mitochondrial membrane potential [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. We also identified the molecular mechanisms mediating these events. Wt p53 protein phosphorylated at Ser^{46} serves as a transcriptional activator of the *p53AIP1* gene [Oda et al., 2000]; the de novo synthesized p53AIP1 protein moves into the mitochondria, promoting dissipation of the membrane potential [Wesierska-Gadek et al., 2005a]. We also identified a cellular kinase that was activated in ROSC-treated MCF-7 cells [Wesierska-Gadek et al., 2007]. When stimulated by ROSC, WT HIPK2 catalyses the phosphorylation of p53, but ATM kinase does not [Wesierska-Gadek et al., 2007]. Similarly, treatment with ROSC prompted the transient expression of wt HIPK2, which enhanced the rate of apoptosis in MCF-7 cells; no such effect was observed in cells with a functionally inactive mutant kinase instead of HIPK2 [Wesierska-Gadek et al., 2007]. These observations clearly indicate that the functional status of p53 determines the efficacy of the therapeutic action of ROSC. However, on the basis of the data available in the literature to date, it is difficult to assess the nature of the functional relationship between the status of p53 and ER-α, as many seemingly contradictory findings have been reported. For instance, it has been reported that wt p53 increases the expression of ER-α in MCF-7 cells [Angeloni et al., 2004]. However, inactivation of p53 in MCF-7 by HPV-encoded E6 oncoprotein reduces the basal levels of ER-α in MCF-7 cells (Fig. 1A). Moreover, in a very recent report it has also been shown that wt p53 participates in *miR*-145 down-regulation of ER-α in breast cancer and in the induction of apoptosis [Spizzo et al., 2010].

In recent years, increasing attention has been paid to the phosphorylation of specific residues of ER-α in breast cancer. The phosphorylation of individual residues within the hormone receptor molecule has been reported to be of prognostic value and seems to determine patients' responses to endocrine therapy. Thus, determination of the phosphorylation status of ER-α might be helpful in identifying breast cancer patients who are likely to benefit from specific endocrine therapies.

CONCLUSIONS

ROSC blocked proliferation of exponentially growing breast cancer cells. However, estrogen-dependent MCF-7 cells were more sensitive to the action of ROSC than other cells. Exposure of MCF-7 cells to ROSC abolished the activating phosphorylation of CDK2, CDK1, and CDK7 in a concentration- and time-dependent manner. Decrease of the site-specific modification of CDK7 correlated with reduction of phosphorylation of ER-α at Ser^{118} and its down-regulation. Furthermore, combining of TAM with ROSC synergistically enhanced the action of the CDK inhibitor in estrogen-dependent MCF-7 cells but not in cells lacking ER-α. Our results indicate that ROSC affects estrogen signaling pathway by several distinct mechanisms making it potentially useful in the treatment of estrogen-dependent breast cancer cells particularly in combination with SERMs.

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