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Roscovitine, a Selective CDK Inhibitor, Reduces the Basal and Estrogen-Induced Phosphorylation of ER-α in Human ER-Positive Breast Cancer Cells

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ABSTRACT

Roscovitine (ROSC), a selective cyclin-dependent kinase (CDK) inhibitor, arrests human estrogen receptor-α (ER-α) positive MCF-7 breast cancer cells in the G_2 phase of the cell cycle and concomitantly induces apoptosis *via* a p53-dependent pathway. The effect of ROSC is markedly diminished in MCF-7 cells maintained in the presence of estrogen-mimicking compounds. Therefore, we decided to examine whether ROSC has any effect on the functional status of the ER-α transcription factor. Exposure of MCF-7 cells to ROSC abolished the activating phosphorylation of CDK2 and CDK7 in a concentration and time-dependent manner. This inhibition of site-specific modification of CDK7 at Ser164/170 prevented phosphorylation of RNA polymerase II and reduced basal phosphorylation of ER-α at Ser118 in non-stimulated MCF-7 cells (resulting in its down-regulation). In MCF-7 cells, estrogen induced strong phosphorylation of ER-α at Ser118 but not at Ser104/Ser106. ROSC prevented this estrogen-promoted activating modification of ER-α. Furthermore, we sought to determine whether the activity of ROSC could be enhanced by combining it with an anti-estrogen. Tamoxifen (TAM), a selective estrogen receptor modulator (SERM), affected breast cancer cell lines irrespective of their ER status. In combination with ROSC, however, it had a different impact, enhancing G_1 or G_2 arrest. Our results indicate that ROSC prevents the activating phosphorylation of ER-α and that its mode of action is strongly dependent on the cellular context. Furthermore, our data show that ROSC can be combined with anti-estrogen therapy. The inhibitory effect of TAM on ER-negative cancer cells indicates that SERMs crosstalk with other steroid hormone receptors. J. Cell. Biochem. 112: 761–772, 2011.

KEY WORDS: ER-α ACTIVATION; P-SER118-ER-α; ESTROGEN; G2 ARREST; CELL CYCLE; ACTIVATION OF WT P53; ANTI-ESTROGEN; TRANSCRIPTIONAL ELONGATION

Estrogen receptors (ERs) are hormone-dependent nuclear transcription factors [Walter et al., 1985; Enmark and Gustafsson, 1999; Matthews and Gustafsson, 2003] that regulate the expression of numerous target genes [Dickson and Stancel, 2000] and mediate many of the biological effects of estrogens in mammary and uterine epithelial cells. Estrogen is a major factor in the development and progression of breast and endometrial cancer; approximately 70% of all breast malignancies express ER-α [Ali and Coombes, 2002]. The majority of these breast tumors are dependent on estrogen signaling [Loi et al., 2007].

ER-α is the major estrogen receptor [Mangelsdorf et al., 1995; Katzenellenbogen and Katzenellenbogen, 1996] and has a number of splice variants, some of which influence the activity and function of its regularly spliced form [Hopp and Fuqua, 1998; Jazaeri et al., 1999]. Like other members of the superfamily, it has a modular structure that encompasses functional domains for ligand- and DNA

Abbreviations used: CDICKI, 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 cell nuclear; PD, Petri dish; PVDF, polyvinylidene difluoride; ROSC, roscovitine; WCL, whole cell lysate; WT, wild-type.

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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; it recognizes and interacts with estrogen-responsive promoters and additionally recruits 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 can be 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 (Ser106 and Ser118) in the amino-terminal AF-1 domain [Joel et al., 1995]. Estradiol has been found to trigger extensive phosphorylation of Ser118 [Joel et al., 1995]. Phosphorylation of this residue seems to be catalyzed by several cellular kinases [for a review, see Lannigan, 2003]. Kato et al. [1995] reported that Ser118 is phosphorylated by MAPK *in vitro* and in COS-1 cells exposed to the EGF and IGF growth factors 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 Ser118 is located in the amino-terminal ligand-independent AF-1 region, it has been shown to be involved in the modulation of estrogen-induced ER-α activation. Mutation of Ser118 markedly reduces transactivation by ER-α [Ali et al., 1993; Le Goff et al., 1994; Kato et al., 1995].

In addition to enhanced activity of ER-α, other perturbations in cellular signaling pathways and/or the proper control of the cell cycle are frequently 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], the inactivation of cellular inhibitors of cyclin-dependent kinases (CDKs) [Sherr and Roberts, 1999; Blagosklonny and Pardee, 2001; Senderowicz, 2001; Negrini et al., 2010] and constitutive activation of the RAS signaling cascade, all of which affect cell cycle regulation, cell proliferation and apoptosis [Nathanson et al., 2001]. 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 two decades, a number of selective CDK inhibitors have been developed [Lapenna and Giordano, 2009; Węsierska-Gądek et al., 2009c; Galons et al., 2010; Rizzolio et al., 2010]. Roscovitine (ROSC), a tri-substituted purine derivative (Seliciclib™; 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, the concentration at which it is used, and the duration of the treatment [Wesierska-Gadek et al., 2009b]. 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 [David-Pfeuty, 1999; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]; it was found to arrest MCF-7 cells at the G_2/M phase of the cell cycle and concomitantly induce apoptosis.

In the present work we sought to determine whether inhibition of CDK7 by ROSC in human MCF-7 breast cancer cells might affect the basal and ligand-induced activity of ER-α, and whether it might be possible to increase its therapeutic utility by using it in conjunction with tamoxifen (TAM), a selective estrogen response modifier (SERM) that interferes with ER-α. 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 inhibited the proliferation of all three tested breast cancer cell lines, albeit with varying degrees of efficiency; MCF-7 cells were more sensitive to its action than were the others. Our results indicate that ROSC modulates estrogen signaling pathways by several distinct mechanisms.

MATERIALS AND METHODS

DRUGS

The purine-derived CDK inhibitor ROSC was obtained from Prof. M. Strnad (Palacky University, Olomouc, Czech Republic). Estradiol (E2), 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), 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), 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 breast 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 [Fan et al., 1995]. 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 and DRB 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), anti-PCNA mouse monoclonal antibody (PC-10) (Santa Cruz Biotechnology, CA), anti-CDK7 (clone MO-1.1) and anti-ER-α (Sigma-Aldrich), rabbit monoclonal anti-phospho Ser118 ER-α (Epitomics, Burlingame, CA), anti-DBC 1, anti-RNA polymerase II phosphorylated on Ser-5 (clone H14) and anti-RNA polymerase II phosphorylated on Ser-2 (clone H5) (all from Abcam plc, Cambridge); 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. 5–7). The effects of the combined ROSC and TAM treatments on the IC_{50} values are shown in Figure 7.

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 (D_m) 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; Schmid et al., 2005]. 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).

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; Schmid et al., 2005]. 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., 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 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.

RESULTS

RAPID ACTIVATION OF THE ER-α TRANSCRIPTION FACTOR IN ESTROGEN-TREATED HUMAN MCF-7 BREAST CANCER CELLS

Initially, the basal level of ER-α transcription factor in different established human breast cancer cells was determined by immunoblotting. Whole cell lysates (WCLs) prepared from untreated exponentially growing cells were loaded on 10% SDS gels (Fig. 1). As expected on the basis of published data, human BT474, T47D, and 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 two of over ten breast cancer cell clones examined (BT-20 and SKBr-3) were ER-α-negative. Further experiments were performed on MCF-7 cells; where appropriate, two ER-α-negative cell lines were used as controls.

To prove the functional status of ER-α, we treated exponentially growing human MCF-7 cells with estrogen (E2) and EGF. Ligands

were applied to cells at a final concentration of 2 or 10 nM, respectively, for 30 and 60 min. Following this treatment, the cells were immediately lysed. As shown in Figure 2, brief exposure of MCF-7 cells to E2 strongly induced phosphorylation of ER-α at Ser118 but not at Ser104/106. Exposure to E2 elevated expression of PCNA (Fig. 2B, 2nd lane). Unlike steroid hormones, EGF only weakly increased the phosphorylation of ER-α at Ser118 (Fig. 2B). No modification of ER-α at Ser104/106 was detected (Fig. 2B, 3rd lane). Treatment with E2 for 30 min was sufficient to markedly activate ER-α; extension of the treatment period to 60 min did not further enhance its activating phosphorylation (Fig. 2A, 3rd lane). Simultaneous co-treatment of MCF-7 cells with E2 and 4-OHT for 60 min decreased the level of phosphorylation of ER-α at Ser118 and elevated its total cellular concentration (Fig. 2A, 4th lane). This seems to be attributable to stabilization of the receptor by 4-OHT. Treatment of MCF-7 cells with E2 and anti-estrogen did not elevate p53 protein in MCF-7 cells. High p53 levels were detected in BT-20 cells used as a positive control (Fig. 2A, 9th lane).

HIGHER BASAL LEVELS OF ER-α IN MCF-7 CELLS CULTIVATED IN PHENOL RED SUPPLEMENTED MEDIUM

Since the responses of MCF-7 cells to different drugs depend on the conditions employed in their cultivation (specifically, on the presence or absence of phenol red in the culture media) [Wesierska-Gadek et al., 2006, 2007b], we also examined the effect of both estrogen and anti-estrogen on MCF-7 cells cultivated in phenol red supplemented medium (Fig. 2A, lanes 5–8). The basal and inducible phosphorylation and protein levels of ER-α were markedly enhanced in MCF-7 maintained in a phenol red-containing medium as compared to cells cultivated in a phenol red-free medium (Fig. 2A, lanes 5–8).

ROSC ABOLISHES BASAL PHOSPHORYLATION OF ER-α IN MCF-7 CELLS

We next sought to determine whether ROSC might affect the functional status of ER-α in MCF-7 cells. For this purpose, cells were exposed to ROSC for 24 h. In keeping with earlier findings, ER-α was weakly phosphorylated at Ser118 but not at Ser104/106 in non-stimulated MCF-7 cells. Exposure of MCF-7 cells to ROSC caused a decrease in the extent of phosphorylation of ER-α at Ser118 in a concentration-dependent manner (40% reduction at 20 μM and 60% at 40 μM; Fig. 3). The total cellular concentration of ER-α decreased by 40% after treatment with 40 μM ROSC. In the latter case, the reduction in ER-α phosphorylation coincided with a strong reduction in the phosphorylation of CDK7 at Ser164/Thr170 (not shown) and of its target, Ser5 of the carboxyl-terminal repeat domain (CTD) of RNA Pol II. Abolition of the site-specific phosphorylation of the CTD is known to cause a global block on transcription. To investigate the possibility that the significant decrease in ER-α phosphorylation upon administration of ROSC is attributable to transcriptional blockage, cells were also treated with DRB, a potent inhibitor of CDK9. Unlike ROSC, DRB reduced neither the phosphorylation of ER-α nor its total cellular levels (Fig. 3, lane 4). Treatment of MCF-7 cells with 4-OHT increased the total cellular concentration of ER-α (Fig. 3; lane 5). With these results in hand, a series of experiments examining the time course of the ROSC-

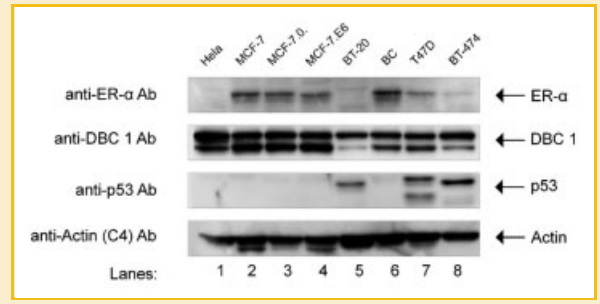

Fig. 1. Determination of ER-α status in a collection of established breast cancer cells lines. WCLs prepared from control human HeLa cervix carcinoma cells and seven breast cancer cell lines were analyzed by immunoblotting after separation on 10% SDS gels. Blots were incubated with antibodies directed 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).

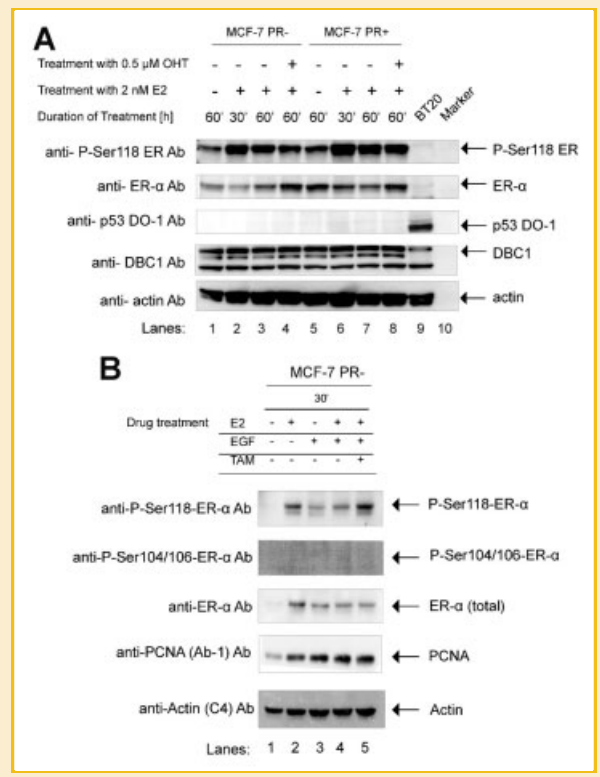

Fig. 2. Ligand-dependent activation of the ER-α transcription factor in human MCF-7 breast cancer cells. A: Determination of a basal and estrogen-inducible phosphorylation of ER-α at Ser118. Human MCF-7 cells maintained in medium with (PR+) and without (PR−) phenol red were collected prior to the onset of treatment and after treatment with estrogen and lysed. WCLs were then analyzed by immunoblotting as described in detail in Figure 1. B: Stimulation of ER-α by estrogen and EGF. Untreated human MCF-7 cells and cells treated with drugs for 30 min as indicated were collected directly after treatment and lysed. WCLs were analyzed by immunoblotting as described in detail in Figure 1. Phosphorylation of ER-α at Ser104/106 and at Ser118 was determined.

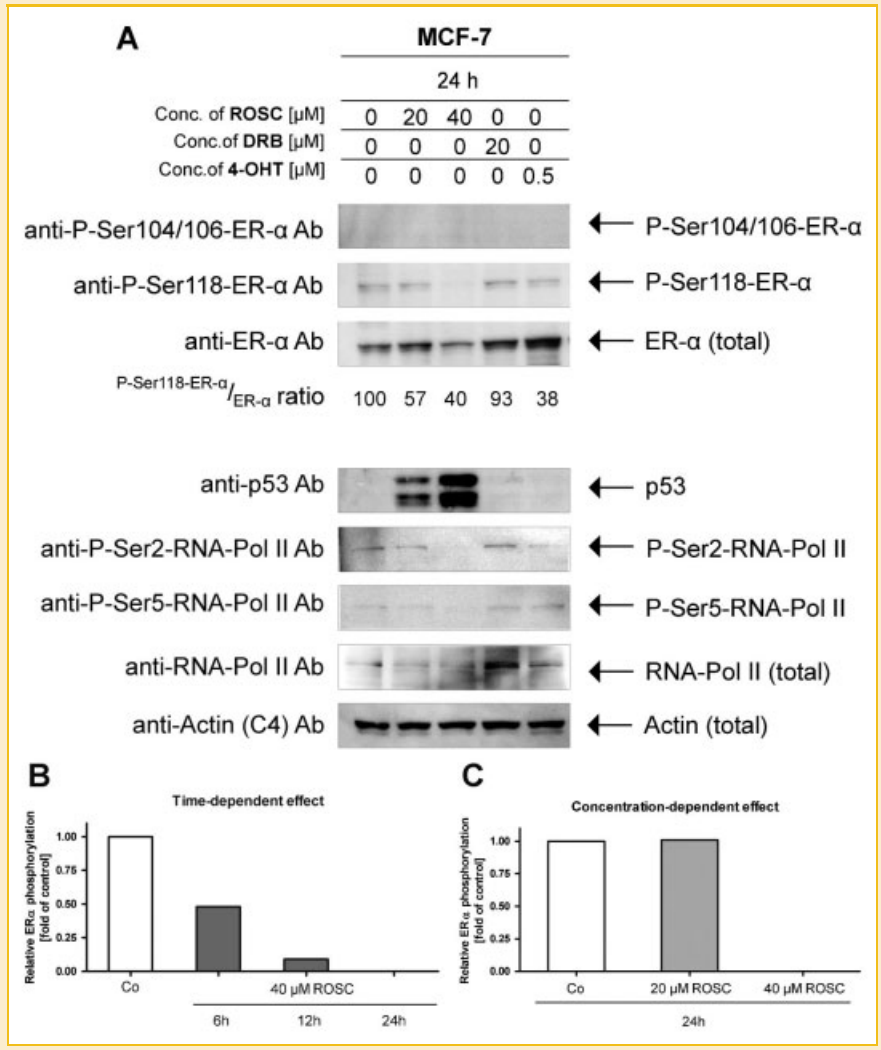

Fig. 3. ROSC but not DRB abolishes basal phosphorylation of ER-α in concentration-dependent manner. A: ROSC decreases phosphorylation of ER-α at Ser118 and reduces its total cellular levels. WCLs prepared from control MCF-7 cells and cells treated with ROSC, DRB, or 4-OHT were separated on 10% or 8% 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). The intensity of protein bands representing P-Ser118 ER-α and total ER-α protein in each lane was normalized against actin. Then P-Ser118 ER-α/ER-α ratio was calculated and normalized against the ratio calculated for the control sample (100). B: Time-dependent decrease of phosphorylation of ER-α in ROSC-treated MCF-7 cells. Relative phosphorylation of ER-α (P-Ser118) calculated for controls and samples after treatment with ROSC for increasing periods of time (6, 12, and 24 h) shown in Figure 4 was plotted in the diagram. C: Dose-dependent decrease of phosphorylation of ER-α in ROSC-treated MCF-7 cells. Relative phosphorylation of ER-α (P-Ser118) calculated for controls and samples after treatment with different doses ROSC shown in Figure 4 was plotted in the diagram.

mediated inhibition of ER-α phosphorylation was performed (Fig. 4). The basal phosphorylation of ER-α at Ser118 was found to decrease in a time-dependent manner when MCF-7 cells were treated with 40 μM ROSC; it was markedly reduced after 6 h and barely detectable after 12 h (Fig. 4). This treatment also strongly induced stabilization of the wt p53 protein, significant quantities of which were observed after only 6 h treatment.

ROSC PREVENTS ESTROGEN-INDUCED ACTIVATION OF ER-α BY PHOSPHORYLATION AT SER118

To determine whether ROSC might be able to counteract the activation of ER-α by E2, control MCF-7 cells and cells treated with ROSC for various periods of time were exposed to E2 for 30 min and then lysed. Pre-incubation of cells with ROSC for 6 h prior to hormone administration did not prevent the ligand-mediated activation of ER-α (Fig. 4A, 5th lane). However, hormone-mediated activation of the receptor was reduced in cells incubated with ROSC for 12 h (Fig. 4A, 8th lane) and suppressed entirely in cells incubated with ROSC for 24 h (Fig. 4B, 7th lane).

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

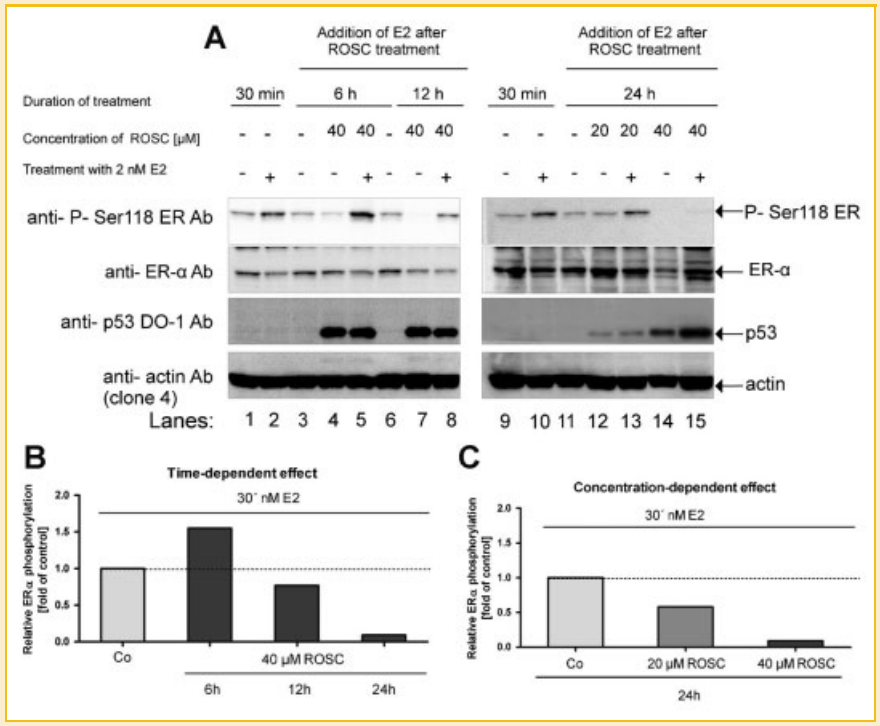

Fig. 4. ROSC prevents ER-α activation in ligand-stimulated MCF-7 cells. A: Higher concentration of ROSC is necessary to prevent estrogen-induced activation of ER-α. MCF-7 cells were incubated for increasing periods of time (30 min, 6, 12, and 24 h) in the presence or absence of ROSC; then E2 was added for 30 min. After termination of treatment cells were lysed. WCLs were analyzed on 10% SDS gels. Blots were incubated with antibodies recognizing ER-α phosphorylated at P-Ser118, against total ER-α and p53 protein. Equal protein loading was confirmed by Ponceau S staining of the membrane and by incubation with anti-actin antibodies. B: Time-dependent decrease of estrogen-induced phosphorylation of ER-α in ROSC-treated MCF-7 cells. Relative phosphorylation of ER-α (P-Ser118) calculated for E2-treated controls and samples after treatment with ROSC for increasing periods of time (6, 12, and 24 h) and short incubation with E2 for 30 min shown in Figure 4A was plotted in the diagram. C: Dose-dependent decrease of estrogen-induced phosphorylation of ER-α in ROSC-treated MCF-7 cells. Relative phosphorylation of ER-α (P-Ser118) calculated for E2-treated controls and samples after treatment with different doses ROSC and short incubation with E2 for 30 min shown in Figure 4 was plotted in the diagram.

plates and exposed to a range of concentrations of ROSC for 24 and 48 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 time- and concentration-dependent manner. Remarkably, ROSC treatment had a much stronger effect on the rate of proliferation of ER-α-positive MCF-7 cells ($IC_{50} = 18.3$ μM) than on that of ER-α-negative BT-20 ($IC_{50} = 22.6$ μM) and SKBr-3 cells (27.2 μM) (Fig. 5).

ROSC INDUCES G_2 ARREST AND SLOWER PROLIFERATION OF MCF-7 CELLS

Exposure of exponentially growing MCF-7 cells to ROSC reduced the numbers of living cells in a time- and concentration-dependent manner (Fig. 6). The analysis of DNA concentrations in single cells revealed that inhibition of cellular CDKs resulted in accumulation of MCF-7 cells arrested in G_2 phase. A short treatment with E_2 had no effect of the cell cycle progression.

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 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. Compared to treatment with ROSC alone, the combination of ROSC with 5 μM TAM had significantly greater inhibitory effects on 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. 7). The interaction between the two compounds was analyzed using CalcuSyn software. The calculated CI was less than one 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 II). The interaction was additive (CI = 1.051) when 40 μM ROSC was combined with 5 μM TAM. Flow cytometric DNA content analysis revealed that the combination of TAM with lower ROSC doses decreased the population of S-phase cells (data not shown).

Interestingly, a weak enhancement of the anti-proliferative effect of ROSC by TAM was also observed in BT-20 (Fig. 7) and SKBr-3 (data not shown) cells lacking ER-α. For these cases, the CIs calculated by interaction analysis were greater than 1, indicating a lack of synergy between TAM and ROSC in ER-α negative breast cancer cells.

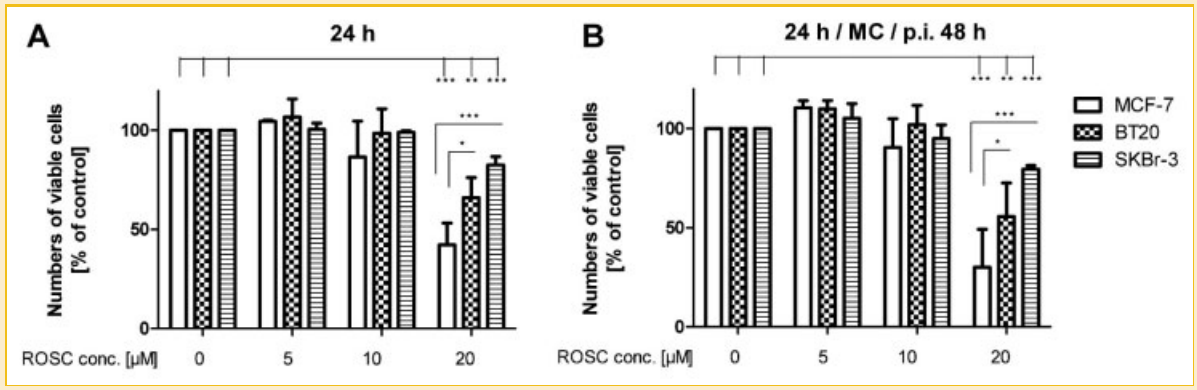

Fig. 5. Different sensitivity of human breast cancer cells to treatment with ROSC. Exponentially growing estrogen-responsive MCF-7 cells and two ER-α-negative cell lines (BT-20 and SKBr-3) were plated in 96-well microtiter plates and 24 h after plating were treated with ROSC at indicated concentrations for 24 h. The numbers of viable cells were determined directly after the treatment (left panel), or alternatively medium was changed and cells were post-incubated in a drug-free medium for 48 h (right panel) using CellTiter-Glo™ assays (Promega Corporation). 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.). Dose–response curves were calculated by nonlinear regression analyses. IC_{50} values determined from dose–response curves are shown in Table I.

DISCUSSION

Breast cancer is the most prevalent malignancy and the second leading cause of cancer-related death in women in developed countries. Over 20 years ago, a hereditary form of breast cancer was identified that is linked to cancer-predisposing germline mutations in genes such as *BRCA1* and *BRCA2* as well as *TP53*, *PTEN*, *STK11*, and others [Nathanson et al., 2001; Da Silva and Lakhani, 2010].

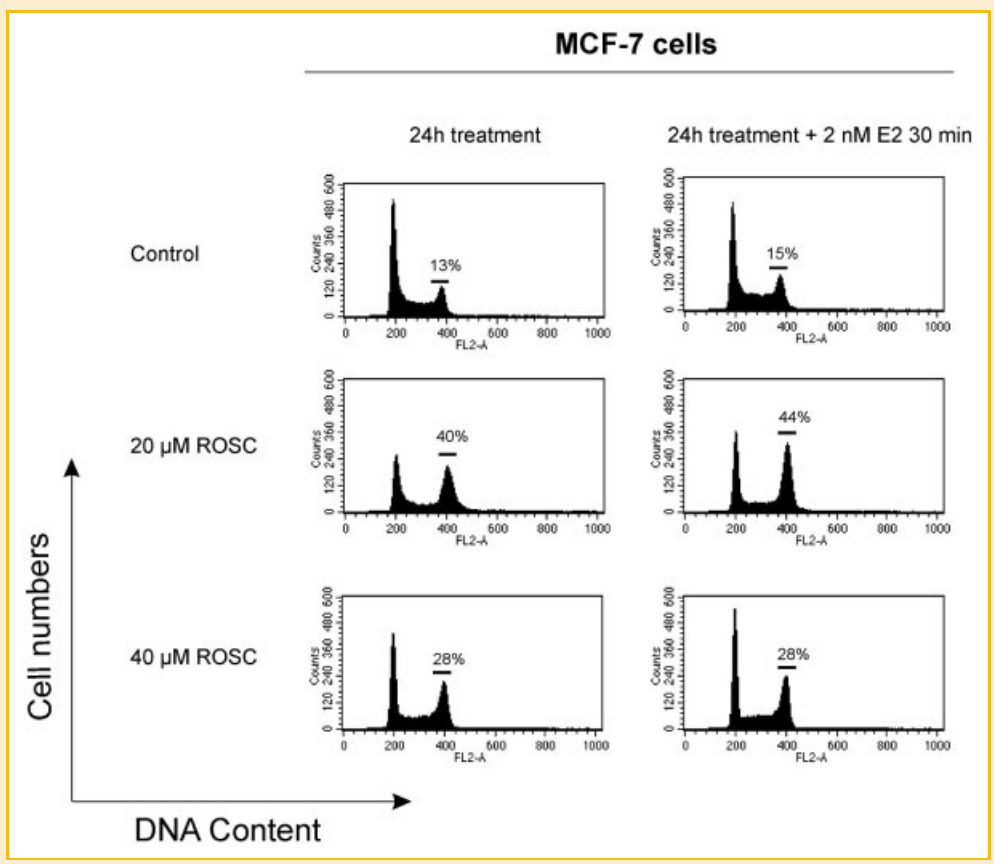

Fig. 6. ROSC induces accumulation of G_2 arrested MCF-7 cells. Exponentially growing human MCF-7 cells were treated with ROSC ($C_E = 20$, 40 µM) for 24 h, or with E2 ($C_E = 2$ nM) for 30 min. Cells were harvested and stained with propidium iodide. DNA content in single cells was measured by flow cytometry. DNA histograms obtained from a representative experiment performed in duplicate were prepared using Cell Quest software. The ratio of G_2 cells is indicated.

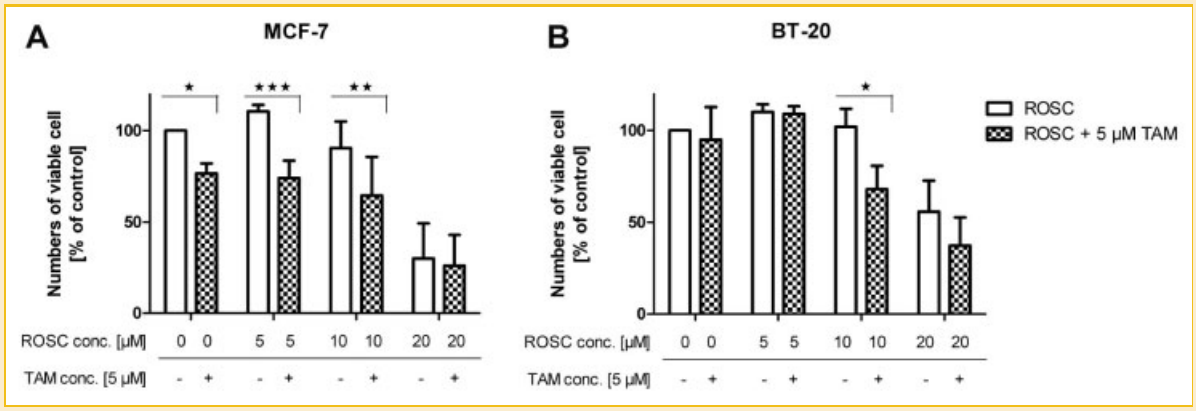

Fig. 7. Interference with ER-α by TAM enhances the anti-proliferative action of ROSC in ER-α-positive breast cancer cells. Exponentially growing MCF-7 cells were plated in 96-well microtiter plates. Twenty-four hours after plating cells were exposed to ROSC alone or ROSC combined with TAM for 48 h in 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). 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.). Statistical significance of the reduction of cell numbers after treatment was calculated using Bonferroni's Multiple Comparison Test. The effect of 5 μM ROSC combined with 5 μM TAM was statistically extremely significant after treatment of MCF-7 cells for 48 h ($P < 0.001$).

Remarkably, inherited predisposition accounts for only 25% of the difference in susceptibility to breast cancer between women with a family history of breast malignancy, indicating that other, non-hereditary factors play an essential role in the carcinogenesis of the breast [Key et al., 2001]. Remarkably, many of the established risk factors are closely linked to estrogenic steroids [Russo and Russo, 1998].

Genomic investigations based on knowledge derived from studies employing traditional biomarkers led to the identification of three major molecular disease groups. The identification of biomarkers is indispensable for prognosis and therapeutic prediction; some biomarkers may even become therapeutic targets in their own right, as was the case with HER2 and ER-α [Perou et al., 2000; Ring et al., 2004].

Breast cancer tumor cells very often display a high mitotic index and reduced apoptotic rate [Yerushalmi et al., 2010]. In keeping with these observations, breast cancer cells exhibit abnormal levels of various factors that regulate apoptosis and overexpress SKP2 (the E3 ubiquitin ligase that targets $p27^{Kip1}$) and cell cycle regulators such as cyclins D_1, E, A, and B1 [Buckley et al., 1993; Bartkova et al., 1994; Signoretti et al., 2002; Davidovich et al., 2008]. Moreover, there is a direct link between estrogenic steroid levels and cell cycle regulation in estrogen-responsive tissues that overexpress ER-α.

TABLE I. Comparison of IC_{50} Values After ROSC Treatment for 24 h

	IC_{50} values	
	24 h	24 h/MC/p.i. 48 h
MCF-7	18.3	16.7
BT-20	22.6	20.4
SKBr-3	27.2	31.8

Cell numbers were determined immediately after 24 h treatment (left column) or alternatively, after medium change and post-incubation (p.i.) (right column) for 48 h in a drug-free medium.

In view of the complex deregulation of the cell cycle observed in breast cancer, the use of selective CDK inhibitors to target overactivated CDK/cyclin complexes is a potentially attractive therapeutic strategy. Indeed, pharmacological inhibitors of CDKs that regulate the early G_1 phase [Finn et al., 2009] and the G_1/S transition [Węsierska-Gądek et al., 2009; Wesierska-Gadek et al., 2010b] have been used in the treatment of established breast cancer cell lines.

ROSC, a tri-substituted purine derivative (Seliciclib™; CYC-202) is a very promising anti-cancer compound that is currently in advanced clinical trials [Le Tourneau et al., 2010]. Although it inhibits several kinases, CDK2 and CDK7 are its primary targets. The biological effects of ROSC are strongly dependent on the type of cell being treated, the concentration applied, the duration of the treatment, and the expression and functional status of a variety of cellular factors involved in the regulation of signal transduction and apoptosis. It is generally accepted that low concentrations of ROSC block cell cycle progression while higher doses induce apoptosis [Wesierska-Gadek et al., 2004a, 2005a, 2008b, 2009a].

The biological effects of ROSC have been particularly extensively studied in human MCF-7 breast carcinoma cells. Compared to healthy cells, MCF-7 cells exhibit a number of abnormalities in

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

	5 μM TAM			
48 h	5 μM ROSC	10 μM ROSC	20 μM ROSC	40 μM ROSC
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 48 h with 5 μM TAM combined with ROSC at indicated concentrations. Combination index (CI) was calculated using CalcuSyn software (Version 2).

terms of the regulation of the cell cycle, signal transduction and apoptosis. Several of these abnormalities render them insensitive to a number of anti-cancer drugs, including: upregulation of ER-α and cyclin D_1; inactivation of the cellular INK4A gene, which encodes both $p16^{INK4A}$ (a cellular CDK inhibitor) and $p14^{ARF}$ (which regulates the interaction between MDM2 and the wt p53 tumor suppressor protein); and loss of caspase-3 activity due to a 47-base pair deletion within exon 3 of the *caspase-3* gene [Fan et al., 1995; Devarajan et al., 2002]. ROSC was found to rapidly arrest cell cycle progression in MCF-7, resulting in an accumulation of cells in the G_2 phase: 6 h treatment was sufficient to induce G_2 arrest in 50% of the tested cells [Wesierska-Gadek et al., 2006]. Interestingly, HIPK2-catalyzed phosphorylation of the p53 tumor suppressor protein at Ser46 was observed after only 3 h exposure to ROSC, resulting in transcriptional induction of the gene encoding the p53AIP1 protein [Wesierska-Gadek et al., 2005a, 2007a]. This protein is translocated into the mitochondria, where it promotes the dissipation of the mitochondrial membrane potential; this in turn prompts the release of apoptosis-inducing mitochondrial proteins such as AIF, APAF-1 and cytochrome *c* into the cytosol and thus triggers apoptosis. However, the course of programmed cell death initiated by ROSC in MCF-7 cells is atypical because in the absence of caspase-3, some important steps in the apoptotic pathway followed by normal cells do not proceed, for example, the activation of caspase-dependent DNAse (CAD), DNA cleavage, and the breakdown of chromatin [Janicke et al., 1998]. Surprisingly, however, the restoration of a functional caspase-3 gene to MCF-7 cells greatly reduced their susceptibility to the action of agents like ROSC that do not damage DNA [Wesierska-Gadek et al., 2010a]; treatment of caspase-3-proficient MCF-7.3.28 cells with ROSC did not result in an increase in the number of cells undergoing apoptosis or cell cycle arrest.

The activity of ROSC has also been studied in other human breast cancer cells with different ER-α and *TP53* statuses. Remarkably, ROSC was preferentially effective in ER-α-positive cancer cells expressing the wt p53 tumor suppressor protein [Gritsch et al., 2011; Zulehner et al., 2011].

The enhanced efficacy of ROSC in breast cancer cells expressing ER-α prompted us to examine its effect on the functional status of the receptor. The activity of ER-α is known to be regulated by post-translational modifications. Phosphorylation of critical residues localized within different functional motifs like LBD, the DNA binding domain (DBD), or AF-1 may change the way ER-α interacts with ligands and other proteins. The AF-1 domain contains serine residues at positions 104 and/or 106, 118, and 167 and plays an important role in regulating the transcriptional function of ER-α. Thus, phosphorylation is part of the downstream signaling cascade initiated by cell surface proteins like EGF, IGF, and PKA [Smith, 1998]. In non-stimulated MCF-7 cells cultivated in phenol red-free media, the basal level of ER-α phosphorylation is very low; phosphorylation is detected at Ser118 but not at Ser104/106. As expected, stimulation of MCF-7 with E2 induced phosphorylation of ER-α exclusively at Ser118, demonstrating that the nuclear steroid receptor is functional in MCF-7 cells. Exposure of non-stimulated exponentially growing MCF-7 cells to ROSC decreased the basal level of ER-α phosphorylation in a time- and concentration-dependent manner; complete suppression of the basal phosphorylation of ER-α at Ser118 was observed after treatment with an elevated concentration of ROSC ($C_E = 40\ \mu M$) for 12 h. In addition, exposure of MCF-7 cells to this high concentration of ROSC for 24 h resulted in the suppression of E2-induced activation of ER-α by site-specific phosphorylation. The prevention of E2-induced activation of ER-α after 24 h coincided with the inhibition of the phosphorylation of CDK7 at Ser164/170 and of Ser5 of the RNA polymerase II (RNA Pol II), which is a substrate of CDK7. Unlike ROSC, the CDK9 inhibitor DRB did not affect the functional status of ER-α in MCF-7 cells. These observations indicate that full inhibition of CDK7 is a key step in the ROSC-mediated suppression of estrogen signaling in MCF-7 cells and explain why prolonged pre-incubations with ROSC are needed to prevent ligand-mediated activation of ER-α [Weitsman et al., 2006].

This concentration-dependent efficacy of ROSC 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 a high mitotic index [David-Pfeuty, 1999; Wesierska-Gadek et al., 2006]. At higher concentrations, ROSC inhibits the activity of CDK7, suppressing the phosphorylation of Ser5 residues within the repetitive motif in the carboxy-terminal domain (CTD) of RNA Pol II. The suppression of this modification prevents the subsequent phosphorylation of Ser2 by CDK9 and thereby 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 on global transcription. The ultimate consequence of this impairment of transcription is largely dependent on the type of cells being treated, the cellular context and the functional status of the major players in the signal transduction and apoptosis pathways. For instance, in human HeLa cervical carcinoma cells, ROSC represses the HPV-encoded E6 and E7 oncoproteins, thereby restoring the G_1/S checkpoint and facilitating the induction of apoptosis [Wesierska-Gadek et al., 2008a, 2008c, 2009b]. Remarkably, by abolishing their site-specific phosphorylation, ROSC changes the functional status of multiple cellular proteins like survivin and Bad [Wesierska-Gadek et al., 2009b]. Importantly, ROSC activates the wt p53 protein, rendering it competent to control or block the progress of the cell cycle, induce apoptosis, and regulate transcription [Wesierska-Gadek et al., 2008c, 2009b].

Finally, we examined the therapeutic potential of combining ROSC-mediated inhibition of cellular CDKs with selective estrogen receptor modulators (SERMs) that interfere with estrogen signaling [Riggs and Hartmann, 2003]. Because estrogenic steroids exert their strong mitogenic effects by activating CDK2/cyclin E complexes, resulting in an acceleration of the G_1/S transition [Fujita et al., 2002], one would expect that ER-positive breast cancer cells with an intact restriction checkpoint would be highly responsive to a combined treatment based on pharmacological interference with the activation of CDK2 and ER-α. Human MCF-7 ER-α-positive breast cancer cells represent suitable testbeds for a combination therapy of this kind, so we employed them in our experiments. The combined treatment strongly enhanced the anti-proliferative effect of ROSC in MCF-7 cells, as demonstrated by the inhibition of the cell proliferation. The interaction between both drugs in MCF-7 cells

was synergistic and potentiated by stimulation with estrogen. However, the interaction between the two drugs was antagonistic in ER-α-negative cells. These results clearly demonstrate the potential of pharmacological approaches based on the simultaneous inhibition of cell cycle progression, ER-α activity, and ER-α-dependent transcription as therapies for the treatment of ER-positive breast cancers.

CONCLUSIONS

ROSC inhibits proliferation of exponentially growing breast cancer cells and is particularly effective against the estrogen-dependent MCF-7 line. It blocks basal and ligand-induced phosphorylation of ER-α at Ser118 in human MCF-7 breast cancer cells. Exposure of MCF-7 cells to ROSC abolished the activating phosphorylation of CDK2, CDK1, and CDK7 in a concentration- and time-dependent manner. This decrease in the extent of the site-specific modification of CDK7 correlated with a reduction in the phosphorylation of ER-α at Ser118. Furthermore, the combination of TAM with ROSC resulted in a synergistic enhancement of the activity 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 pathways 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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