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Inhibition of Farnesyl Protein Transferase Sensitizes Human MCF-7 Breast Cancer Cells to Roscovitine-Mediated Cell Cycle Arrest

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Abstract We reported recently that roscovitine (ROSC), a selective cyclin-dependent kinase (CDK) inhibitor, arrests human MCF-7 breast cancer cells in G_2 phase of the cell cycle, and concomitantly induces apoptosis. Human MCF-7 breast cancer cells are known to express elevated levels of c-Ha-Ras protein. To achieve full biological activity, *de novo* synthesized c-Ha-Ras protein has to be farnesylated and after further processing it needs to be attached to the plasma membrane. Therefore, we decided to prove whether prevention of protein farnesylation would sensitize MCF-7 cells to the action of ROSC. MCF-7 cells were treated with 1–40 μM ROSC alone, or in combination with L-744,832, an inhibitor of farnesyl protein transferase (FTPase). To measure the impact on the proliferation of the cells, we used the CellTiterGlo™ viability assay and FACS analysis was employed to quantify the DNA-content of the single cells. The amount and phosphorylation status of relevant proteins after lysis of MCF-7 cells was assessed on Western blots using (phospho)-specific antibodies. The combined treatment with L-744,832 and ROSC for 24 h, markedly reduced the number of viable MCF-7 cells, primarily, by re-enforcing the cell cycle arrest. Interestingly, the potentiation of the ROSC-mediated inhibition of cell proliferation became evident during the 48 h post-incubation period in presence of the FPTase inhibitor. Inhibition of FPTase in ROSC-treated cells reduced the number of viable cells by approximately 30%. Evidently, the combined treatment sensitizes MCF-7 cells to the action of ROSC, thereby allowing to reduce the dose of the drug and to minimize side effects. J. Cell. Biochem. 102: 736–747, 2007.

Key words: G_2 cell cycle arrest; CDK-inhibitors; p53 activation; signal transduction

A stringent control of the cell cycle progression is a prerequisite for growth and, if necessary, growth arrest of normal cells [van den Heuvel and Harlow, 1993; Nurse, 1997, 2002; Jacks and Weinberg, 1998]. One possible detrimental consequence of an escape of eukaryotic cells from the correct cell cycle regulation can be the induction of malignant transformation [Weinberg, 1996; Vogelstein and Kinzler, 2004].

This malignant transformation of cells that occurs during the development of cancer [Motokura and Arnold, 1993; Rodriguez-Puebla et al., 1999] is characterized by inactivating mutations or by alterations in the expression and activity of proteins involved in the proper regulation of the cell cycle and by disturbances in the induction of apoptosis [Weinberg, 1996; Nurse, 2002; Vogelstein and Kinzler, 2004]. Unfortunately, most changes that lead to an accelerated growth of a cell establish a selective evolutionary advantage for this particular cell. Therefore, a tight control of cellular growth and susceptibility of cells to respond to pro-apoptotic stimuli is of outstanding importance for multicellular organisms to prevent the uncontrolled

Abbreviations used: CDKI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; FPTase, farnesyl protein transferase; FTI, farnesyl protein transferase inhibitor; MDM-2, mouse double minute-2; MCM7, minichromosome maintenance 7; p53AIP-1, p53 apoptosis inducing protein 1; PARP-1, poly(ADP-ribose) polymerase-1; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; ROSC, roscovitine; WCL, whole cell lysate; WT, wild-type.

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growth of mutated cells. For this purpose, cells have acquired a fine-tuned system of control mechanisms such as tumor suppressor genes and endogenous inhibitors of cyclin-dependent kinases (CDKs) that regulate the cell cycle progression [Sherr and Roberts, 1999; Senderowicz, 2003].

The p53 protein, a product of a tumor suppressor gene is one of the key molecules controlling the cell cycle machinery and the induction of apoptosis and is therefore, a prime target for anti-cancer therapy [Prives and Hall, 1999; Bargonetti and Manfredi, 2002; Blagosklonny, 2002; Toledo and Wahl, 2006]. It has been recognized that the (re)activation of wt p53 in cancer cells may substantially contribute to the outcome of a chemotherapeutic intervention [Blagosklonny, 2002; Wesierska-Gadek et al., 2002]. Wt p53, usually maintained in cells at low levels, is upregulated by a variety of anti-cancer drugs, what is most frequently attributable to DNA damage generated by these agents [Blagosklonny, 2002; Wesierska-Gadek et al., 2002]. Interestingly, a number of newly developed inhibitors of CDKs, such as flavopiridol, UCN-01, or substituted purines, additionally to their CDK2 inhibiting properties, also positively affect the levels of p53 protein, given that these tumor cells express a functional p53 protein [David-Pfeuty, 1999; Kotala et al., 2001; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2006a]. Interestingly, ATP-mimicking substituted purines like roscovitine (ROSC) or olomoucine [De Azevedo et al., 1997; Meijer et al., 1997; Gray et al., 1999] show only negligible, if any, direct cytoxicity but activate the p53 protein in a fashion not depending on damaged DNA [Ljungman and Paulsen, 2001; Wesierska-Gadek et al., 2005a]. ROSC is a very efficient drug concerning human MCF-7 breast cancer cells and it inhibits proliferation of MCF-7 cells at lower concentrations than cisplatin [Wesierska-Gadek et al., 2003]. ROSC arrests naive, asynchronously growing MCF-7 cells in the G_2 phase of the cell cycle [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a], whereas the cells released from a nocodazole-mediated mitotic block, accumulate in G_1 phase [David-Pfeuty, 1999]. Moreover, longer exposure of cells to ROSC results in the induction of apoptosis [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Surprisingly, the induction of apoptosis occurred after exposure of asynchronously growing MCF-7 cells to ROSC treatment, but not in cells released from a nocodazole-mediated mitotic block [David-Pfeuty, 1999]. Careful comparison of the experimental conditions revealed that the latter were cultivated in conventional medium. This finding raised the question whether components of the media themselves could modulate the anti-proliferative and/or pro-apoptotic action of ROSC on MCF-7 cells.

A recent report [Wesierska-Gadek et al., 2006b] showed that phenol red has a growth-promoting effect on MCF-7 cells. However, within the first 24 h after plating of cells, the difference in the growth kinetics was relatively low, implicating that this time window is suitable for experiments designed to evaluate the efficacy of a therapeutic intervention with CDK inhibitors, when used for breast cancer cells. Interestingly, the impact of ROSC was negatively affected by supplementation of the medium with phenol red. ROSC inhibited proliferation of breast cancer cells more efficiently when cultivated in phenol red-deprived medium. In concordance with our previous observations, exposure of MCF-7 cells to ROSC resulted in a G_2 arrest. However, the kinetics and the extent of the G_2 block strongly depended on the composition of the medium. Approximately, 50% of cells cultivated in phenol red-deprived medium were G_2-arrested after exposure to ROSC for 12 h, whereas only 30% of cells cultivated in medium supplemented with phenol red were accumulated in G_2 [Wesierska-Gadek et al., 2006b]. The clearly weaker effect of ROSC on the cell cycle in cells cultivated in the presence of phenol red coincided with a lack of CDK2 inhibition [Wesierska-Gadek et al., 2006b]. Moreover, the number of cells undergoing apoptosis after longer exposure to ROSC was significantly reduced, when cells were cultivated in phenol red-supplemented medium. These results evidence that phenol red modulates the therapeutic effect of ROSC on MCF-7 cells by accelerating their proliferation and by reducing their susceptibility to pro-apoptotic stimuli.

ROSC induced a prolonged cell cycle arrest in MCF-7 cells. After post-incubation in a drug-free medium for 24 h [Wesierska-Gadek et al., 2005a], the frequency of G_2-arrested cells was not diminished, but the number of viable cells was markedly reduced. This observation indicates that after exposure of cancer cells to the mentioned drugs at lower concentrations,

their curative effect becomes detectable after a longer interval and that this offers a time window for additional targeting of distinct pathways that are constitutively activated in malignant cells. In the present article, we examined the combined action of ROSC and L-744,832. We observed that the simultaneous treatment with ROSC and L-744,832 for 24 h, markedly reduced the number of viable MCF-7 cells, primarily, by re-enforcing the cell cycle arrest. Interestingly, the potentiation of the ROSC-mediated inhibition of cell proliferation became more evident during the 48 h post-incubation period in presence of the FPTase inhibitor. These results clearly indicate that the combined treatment sensitizes MCF-7 cells to the action of ROSC.

MATERIALS AND METHODS

Drugs

We used ROSC, a selective inhibitor of CDKs and inhibitor of FPTase: the farnesyltransferase inhibitor (2S)-2-[(2S)-2-[(2S, 3S)]-2-[(2R)-2 Amino-mercapthopropyl]aino]-3-methylpenthyl] oxy]-1-oxo-3-phenylpropyl]amino]-4-(methylsyulfonyl)-butanoic Acid 1-Methylethyl Ester (L-744,832) from Calbiochem (Calbiochem-Novabiochem Corp., La Jolla, CA). Stock solutions of the drugs ROSC and L-774,832 were prepared in DMSO. Aliquots of stock solutions were protected from light and stored until use at -20°C.

Cells and Treatment

Human MCF-7 breast carcinoma cells were grown as a monolayer in Dulbecco's medium without phenol red supplemented with 10% FCS at 37°C in an atmosphere of 8% CO_2 [Wesierska-Gadek et al., 1996, 2006b]. Cells were grown up to 60% confluence and then treated with ROSC at a final concentration ranging from 1–40 μM for indicated periods of time. In some experiments cells were treated additionally with L-744,832, an inhibitor of farnesyl protein transferase (FPTase) at a final concentration of 25 μM. Rat embryo fibroblasts transformed with ts $p53^{135val}$ + c-Ha-ras were used as a positive control [Wesierska-Gadek et al., 1996, 2006b]. The establishment of transformed rat cell clones was described previously in detail [Wesierska-Gadek et al., 1996].

Antibodies

We used the following antibodies: monoclonal anti-p53 antibody DO-1, a kind gift from Dr. B. Vojtesek (Masaryk Memorial Cancer Institute, Brno). The monoclonal anti-MCM7 (clone DCS141.2) and anti-PCNA (PC-10) antibodies were from Oncogene Research Products (Cambridge, MA). Polyclonal anti-phospho-Thr160 CDK2 and corresponding antibodies against the total antigen were from New England Biolabs (Beverly, MA). Monoclonal antibodies against p21-c-Ha-Ras (clone NCC-Ras-001) were from DAKO A/S (Glostrup, Denmark), and anti-ran antibodies were from Transduction Laboratories (Lexington, KY). Monoclonal anti-actin (Clone C4) antibodies were from ICN Biochemicals (Aurora, OH). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from Amersham International (Little Chalfont, Buckinghamshire, UK).

Determination of the Number of Viable Cells

Proliferation of human MCF-7 breast cancer cells and their sensitivity to increasing concentrations of ROSC was determined by the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). As described recently in more detail [Wesierska-Gadek et al., 2005b], the CellTiter-Glo™ Luminescent Cell Viability Assay, generating luminescent signals, is based on quantification of the cellular ATP levels. Tests were performed at least in quadruplicates. Luminescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter. Each point represents the mean ± SD (bars) of replicates from at least four experiments.

Measurement of DNA of Single Cells by Flow Cytometry

Measurement of the DNA-content was performed by flow cytometric analysis based on a slightly modified method [Wesierska-Gadek and Schmid, 2000] described previously by Vindelov et al. [Vindelov et al., 1983]. The cells were detached from substratum by limited trypsinization, then all cells were harvested by centrifugation and washed in PBS. Aliquots of 10^6 cells were used for further analysis. Cells were stained with propidium iodide as described previously and then the fluorescence was

measured using the Becton Dickinson FACScan after at least 2 h incubation at +4°C in the dark.

Cell Fractionation

Cells were harvested, washed with PBS, and resuspended in ice-cold, hypotonic buffer supplemented with protease inhibitors [Wesierska-Gadek et al., 2006a]. After swelling of cells in hypotonic buffer without addition of detergents and after disruption of the integrity of plasma membrane, soluble cytosolic proteins were separated from the high molecular weight cellular structures by a centrifugation step at 5,000 × g for 15 min at +4°C. The supernatant was designated as cytosol and the pellet as membrane-bound (MB) fraction. Subcellular fractions were collected and analyzed.

Electrophoretic Separation of Proteins and Immunoblotting

Total cellular proteins or proteins of the distinct subcellular fractions dissolved in SDS sample buffer were separated on 10 or 15% SDS slab gels, transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF) (Amersham International, Little Chalfont, Buckinghamshire, England), and immunoblotted as previously described [Wesierska-Gadek et al., 1995]. Equal protein loading was confirmed by Ponceau S staining. To determine the phosphorylation status of selected proteins, antibodies recognizing site-specifically phosphorylated proteins were diluted to a final concentration of 1:1,000 in 1% BSA in Tris-saline-Tween-20 (TST) buffer. When necessary, blots were used for sequential incubations.

RESULTS

Exposure of MCF-7 Cells to ROSC Inhibits Cell Proliferation

In agreement with previously published data [Eckert et al., 2004], treatment of human MCF-7 cells for 24 h with increasing ROSC concentrations resulted in a marked reduction of the number of viable cells in a time- and concentration-dependent manner (Fig. 1). After administration of ROSC, *in vivo* (treatment of patients) cancer cells are exposed to a high concentration of the drug for approximately 24 h and then, due to a drug clearance, its concentration decreases [de la Motte and Gianella-Borradori, 2004]. To mimic in cell culture, these in vivo conditions occurring during the treatment of patients, we

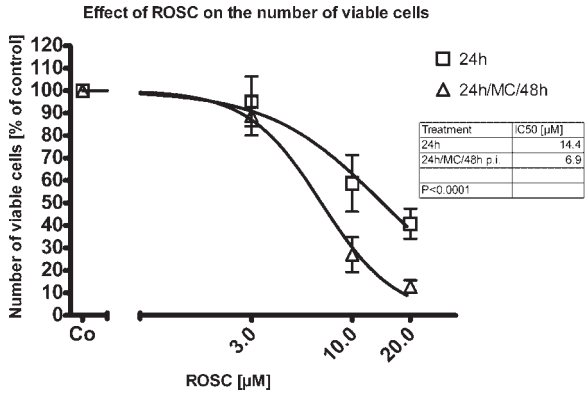

Fig. 1. Reduction of the number of viable MCF-7 cells after treatment with ROSC. Exponentially growing MCF-7 cells plated in 96-well microtiter plates were treated for 24 h with increasing concentrations of ROSC. The number of viable cells was determined directly after treatment (open square 24 h) and additionally, after post-incubation for 48 h in a drug-free medium (open triangle 24 h/MC/48 h). The graph represents mean values ± SD from five independent experiments, each performed at least in quadruplicates. $IC_{50} = 14.37$ μM ROSC after 24 h treatment; $IC_{50} = 6.93$ μM ROSC after treatment for 24 h, medium change and post-incubation for 48 h. The difference of IC_{50} values is statistically very highly significant ($P < 0.0001$).

exposed MCF-7 cells to increasing ROSC concentrations for 24 h and then, following medium change, cells were post-incubated in a drug-free medium for a further 48 h. The comparison of the action of ROSC directly after 24 h treatment and 48 h post-incubation revealed a great difference between short- and long-term effects of ROSC treatment (Fig. 1). The IC_{50} calculated from values measured directly after 24 h treatment with ROSC was 14.4 μM, and it decreased approximately two-fold after post-incubation for 48 h (6.9 μM ROSC). These results implicate that viable cells also, after 24 h treatment with lower ROSC doses, remained arrested or were even triggered to die. This assumption became evident during the post-incubation period. Cells were unable to divide or they even died during cultivation for another 48 h. These results also indicate that the post-incubation time window offers an excellent opportunity for a treatment with non-genotoxic agents to inhibit signal transduction processes and/or cell cycle progression, thereby enhancing the primary effect of ROSC.

The Effect of ROSC on the Cellular Level of c-Ha-Ras in MCF-7 Cells

Cancer cells frequently express increased levels of c-Ha-Ras protein. The comparison of

c-Ha-Ras expression between a few frequently used human cell lines revealed a strong c-Ha-Ras protein band in human MCF-7 and HepG2 cancer cells (Fig. 2). Lysates from transformed rat cells overexpressing mutated p53 and human c-Ha-Ras were loaded as a positive control. Since ROSC inhibits not only the activity of cell cycle kinases but also that of RNA polymerase II [Ljungman and Paulsen, 2001], resulting in a block of global transcription, one cannot exclude the possibility that the long exposure of MCF-7 cells (for 15 and 24 h) could affect the level of cellular c-Ha-Ras protein. Therefore, we examined the effect of ROSC on the expression of c-Ha-Ras protein. As shown in Figure 3, exposure of MCF-7 cells for 24 h to 3 and 10 μM ROSC did not affect the total cellular amount of c-Ha-Ras protein. However, according to the predictions, the combined treatment with ROSC and FTI resulted in a shift of the reactive c-Ha-Ras protein band (Fig. 3) that reflects the alteration of its electrophoretic mobility due to lack of processing.

Inhibition of FPTase Prevents the Attachment of c-Ha-Ras Protein to the Plasma Membrane

To assess the effect of the drugs alone, or in combination, the subcellular distribution of c-Ha-Ras protein, untreated MCF-7 cells (as control), and cells exposed to L-744,832 for 24 h,

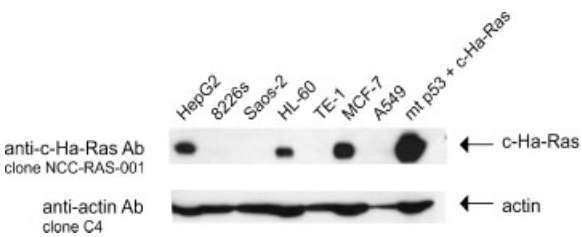

Fig. 2. MCF-7 cells express high levels of c-Ha-Ras protein. Untreated, exponentially growing human cancer cell lines were harvested and lyzed. Whole cell lysates (WCLs) were loaded on 15% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The cellular level of c-Ha-Ras protein was determined by immunoblotting using a monoclonal anti-c-Ha-Ras antibody and protein loading was checked by sequential incubation with anti-actin antibodies. The following human cancer cells were analyzed: hepatic carcinoma HepG2, 8226 myeloma [Wesierska-Gadek, 2005], osteosarcoma Saos-2, promyelocytic leukemia HL-60, esophageal cancer cell line TE-1 $p53^{V272M}$ [North et al., 2002], and lung adenocarcinoma A549 cells overexpressing Ki-Ras. WCL prepared from transformed rat cells expressing mutant p53 + c-Ha-Ras were loaded as a positive control [Wesierska-Gadek et al., 2006a].

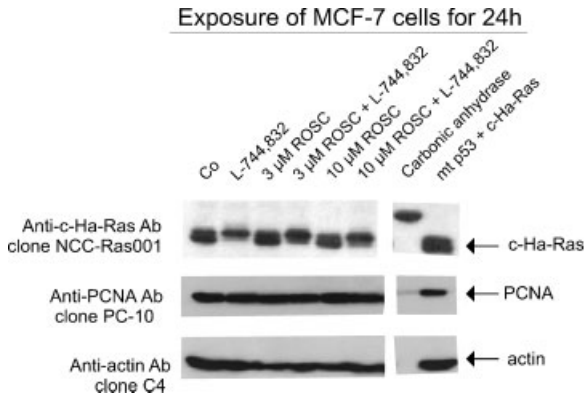

Fig. 3. Exposure of MCF-7 cells to ROSC did not affect the cellular levels of c-Ha-Ras protein. Exponentially growing MCF-7 cells were treated for 24 h with ROSC alone, or in combination with the FPTase inhibitor L-744,832 (FTI) at a final concentration of 25 μM, and then lyzed. WCLs were loaded on 15% SDS-gels. Conditions of separation and immunoblotting were described in detail in Figure 2. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

alone or in combination with ROSC, were fractionated. Cells were suspended in ice-cold hypotonic medium without any detergents and allowed to swell. Soluble cytosolic proteins were separated from the high molecular weight cellular structures by a single centrifugation step. The fractionation revealed that in untreated MCF-7 cells (control) c-Ha-Ras protein was almost completely integrated in the plasma membrane and no soluble form could be detected in the cytosol (Fig. 4). After exposure of MCF-7 cells to 10 μM ROSC for 24 h a small portion of c-Ha-Ras became soluble and was detected in the cytosolic fraction (Fig. 4). However, after treatment for 24 h with L-744,832 alone, or in combination with ROSC, the majority of c-Ha-ras protein became soluble and it was found in the cytosolic fraction (Fig. 4). The blots were sequentially incubated with anti-ran antibodies to prove the equal protein loading. Unlike actin, ran protein shuttles between two major cellular compartments, the cytoplasm and the nucleus, and is therefore suitable to confirm loading of the proteins of the soluble fraction obtained after a high-speed centrifugation step.

L-744,832 Combined with ROSC Strongly Reduces the Number of Viable Cells

Considering the fact that MCF-7 cells overexpress c-Ha-Ras protein and that the inhibition of FPTase results in the reduction of the

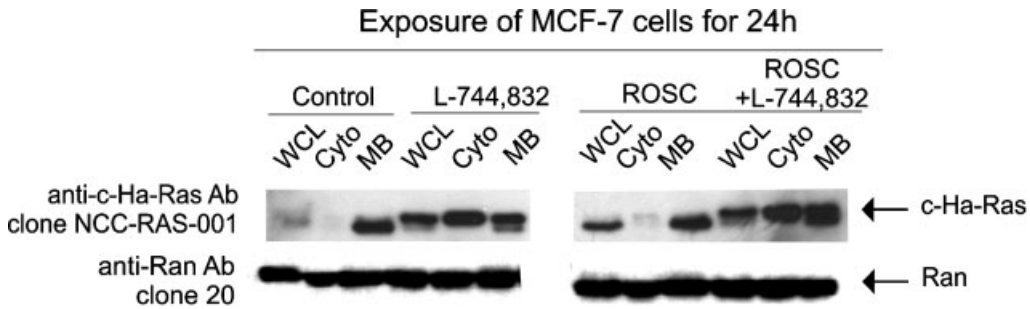

Fig. 4. Loss of the attachment of de novo synthesized c-Ha-Ras protein to plasma membrane after treatment of MCF-7 cells with inhibitor of FPTase. MCF-7 cells treated for 24 h with the FPTase inhibitor L-744,832, or with ROSC alone, or in combination with FTI, were collected and fractionated to separate the soluble (cytosol) and MB form of c-Ha-Ras protein. Proteins were loaded on 15% SDS-gels. Conditions of separation and immunoblotting were described in detail in Figure 2. Equal loading was checked by sequential incubation with anti-Ran antibodies. Ran protein shuttling between nucleus and cytosol is suitable to prove the protein loading of these fractions.

adequate localization of c-Ha-Ras protein in MCF-7 cells, we determined the effect of FPTase inhibitors alone, or in combination with ROSC, on the cell proliferation. Exposure of MCF-7 cells to a low ROSC concentration (3 μM) for 24 h did not reduce the number of viable cells (Figs. 1 and 5). After treatment with the FPTase inhibitor L-744,832 for 24 h at a final concentration of 25 μM, the number of viable cells was diminished by 20% (Fig. 5A). The combined treatment with 3 μM ROSC and L-744,832 for 24 h only slightly affected the viability of the cells as compared with the action of FPTase inhibitor alone. However, simultaneous administration of L-744,832 and 10 μM ROSC reduced the number of living cells by 65%, approximately 20% more as compared with ROSC alone. The decrease of the cell number was statistically highly significant (Fig. 5B). After combined treatment for 24 h, the IC_{50} value of ROSC was approximately twofold reduced. This reduction was statistically significant (Fig. 5A).

L-744,832 Enhances ROSC-Mediated Cell Cycle Arrest at the G_2 Phase and Reduces the Proportion of the S-phase Population

ROSC affects the cell cycle in a concentration-dependent manner. The exposure of human MCF-7 cells to ROSC at a low concentration (3 μM) for 24 h did not affect cell cycle

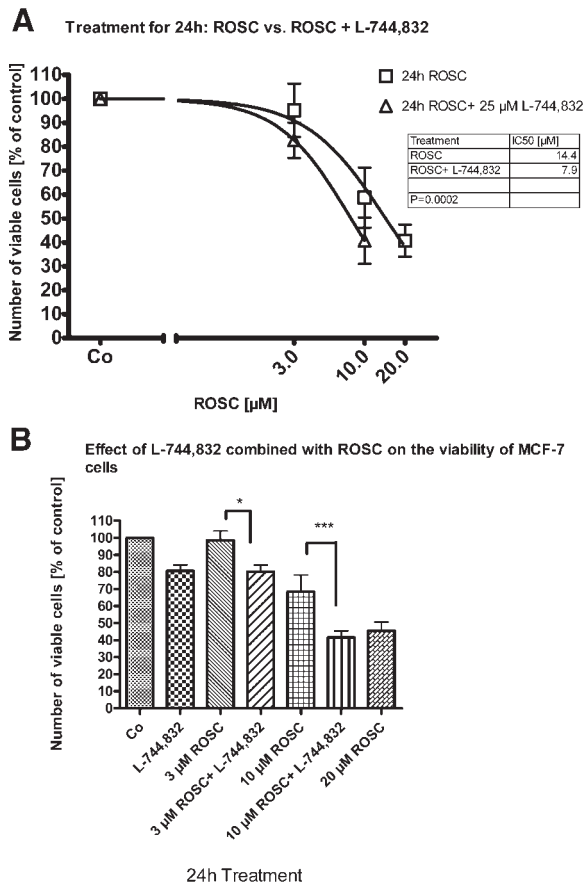

Fig. 5. Inhibition of FPTase synergistically affects the ROSC-mediated reduction of the number of viable MCF-7 cells. **A**: MCF-7 cells were treated for 24 h with ROSC alone (open square), or in combination with L-744,832 ($C_E = 25$ μM) (open triangle). The number of viable cells was determined directly after treatment using the CellTiterGlo Assay. **B**: Statistical evaluation of the effect of 25 μM L-744,832 combined with low ROSC doses on the viability of MCF-7 cells. The differences between values obtained after 10 μM ROSC alone, or in combination with the FTI, are statistically very highly significant ($P < 0.001$). The exposure of MCF-7 cells for 24 h to 25 μM L-744,832 alone reduced the number of viable cells by approximately 20%. The decrease was not statistically significant.

progression. However, after treatment with 10 μM ROSC, the frequency of G_2-arrested cells markedly increased (Fig. 6). On the other hand, L-744,832 administered alone augmented the number of cells in G_1. The combined treatment re-enforced ROSC-mediated cell cycle inhibition at G_2. This effect became more evident after comparison of the S/G_1 and G_2/G_1 ratios.

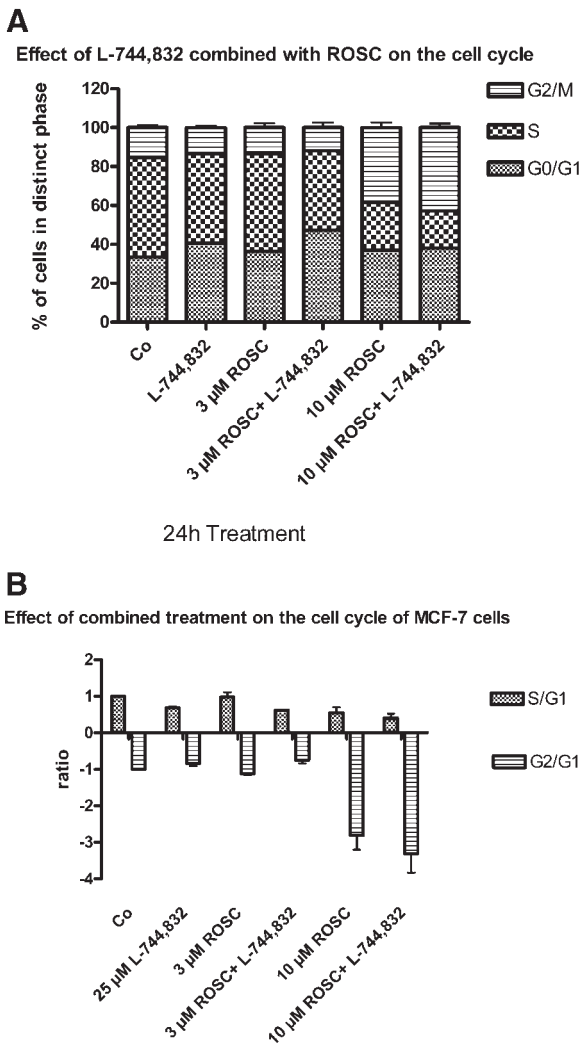

Fig. 6. Combined treatment of MCF-7 cells with ROSC and an inhibitor of FPTase for 24 h increases the population of G_2-arrested cells. MCF-7 cells treated with drugs for 24 h as indicated were detached from substratum by limited trypsin digestion and single cell suspension was used for propidium iodide staining. DNA-content in single cells was measured using the Becton Dickinson FACScan flow cytometer. **A**: The distribution of MCF-7 cells in distinct cell cycle phases. The values represent the mean of three replicates. **B**: Diagram showing the changes of S/G_1 and G_2/G_1 ratios after treatment. The values represent the mean of replicates from three experiments.

Exposure of MCF-7 Cells to L-744,832 During the Post-Incubation Period Strongly Enhances ROSC-Mediated Inhibition of the Cell Cycle and Cell Proliferation

It became obvious that the exposure of MCF-7 cells to inhibitors of FPTase during post-incubation for 48 h strongly diminished the ratio of living MCF-7 cells (Fig. 7). The reduction of the IC_{50} values and of the number of viable cells was statistically very highly significant (Fig. 7A and B). Moreover, the inhibition of FPTase during post-treatment of MCF-7 cells for 48 h strongly affected their distribution in distinct cell cycle phases (Fig. 8A). The frequency of the S-phase population was

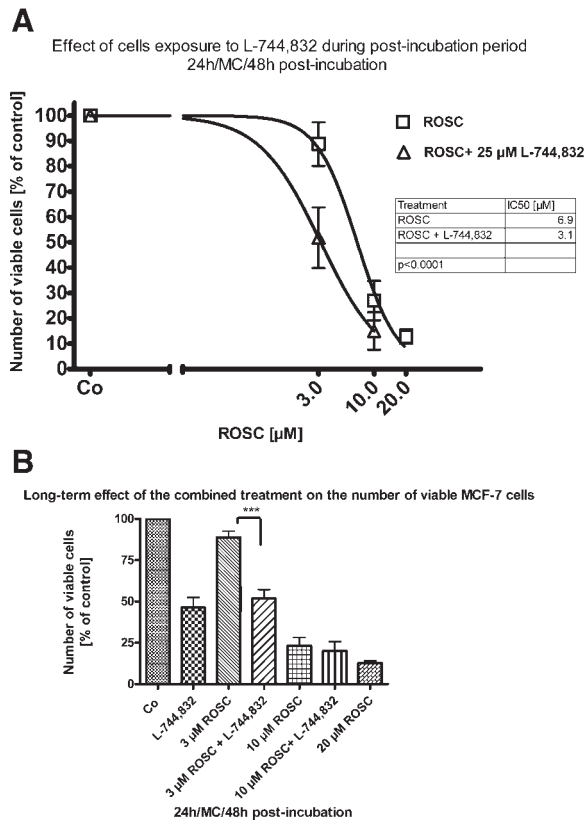

Fig. 7. Inhibition of FPTase synergistically enhances the anti-proliferative action of ROSC. MCF-7 cells were treated for 24 h with ROSC alone (open square), or in combination with L-744,832 (open triangle). After 24 h treatment, medium was changed and cells were post-incubated in a drug-free medium or in the presence of L-744,832 for 48 h. The number of living cells was determined using the CellTiterGlo Assay. Combined treatment reduced the IC_{50} value more than twofold (6.9 vs. 3.1 μM ROSC). The IC_{50} decrease was statistically very highly significant ($P < 0.0001$). **B**: The differences between values obtained after 3 μM ROSC alone or in combination with the FTI are also statistically very highly significant ($P < 0.001$).

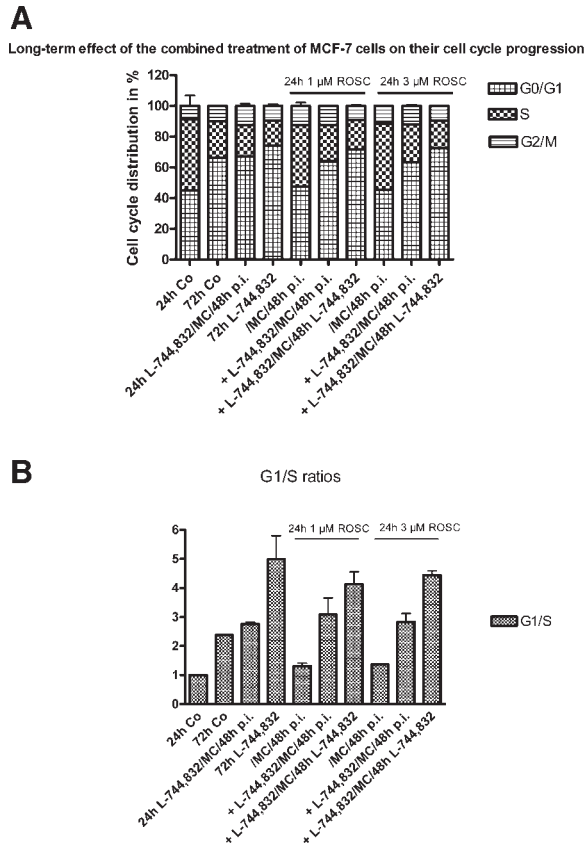

Fig. 8. Prevention of farnesylation markedly increases the population of G_1 arrested MCF-7 cells. MCF-7 cells treated with drugs for 24 h and then post-incubated as indicated. Conditions of cell harvesting and staining as in Figure 6. **A**: The distribution of MCF-7 cells in distinct cell cycle phases. **B**: Diagram showing the changes of G_1/S ratio after treatment. The values represent the mean of replicates of three independent experiments.

markedly reduced. This was accompanied by an obvious G_1 arrest (Fig. 8A and B). The increase in the G_1/S ratio became evident already at 1 µM ROSC (Fig. 8B). The accumulation of G_1-arrested cells observed after the inhibition of FPTase during post-treatment of MCF-7 cells for 48 h strongly correlates with the reduction of the number of viable cells.

L-744,832 Synergistically Increases ROSC-Mediated Induction of wt p53 Protein in MCF-7 Cells

ROSC is known to positively affect the stability of wt p53 protein in a time- and concentration-dependent manner. Surprisingly, after exposure of MCF-7 cells to L-744,832 for 24 h, the cellular level of wt p53 protein in MCF-7 cells increased. The combined treatment with ROSC enhanced the p53 expression more strongly than ROSC alone (Fig. 9). The upregulation of wt p53 protein after combined treatment could be of functional importance because low ROSC concentrations were not sufficient to inhibit the activating phosphorylation of CDK2. As shown in Figure 10, ROSC, even at a final concentration of 10 µM, did not completely abolish the site-specific phosphorylation of CDK2. Therefore, the increase of wt p53 could negatively affect the cell cycle progression in a different way.

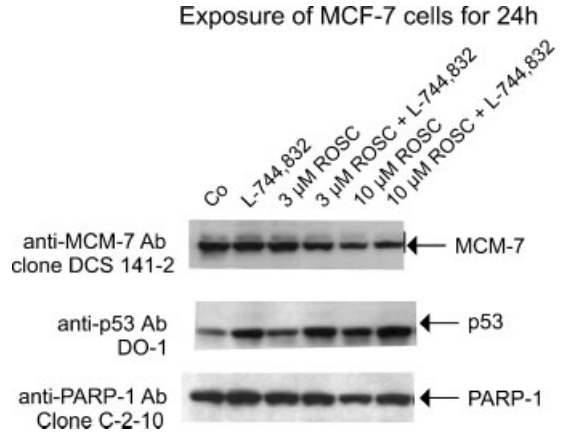

Fig. 9. L-744,832 enhances ROSC-mediated induction of p53 protein in MCF-7 cells. Exponentially growing MCF-7 cells were treated for 24 h with ROSC alone, or in combination with the FTI, and then lyzed. WCLs were loaded on 10% SDS-gels. Conditions of separation and immunoblotting were described in detail in Figure 2. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

DISCUSSION

The Ras protein is mutated or upregulated in a wide variety of tumor cells and typically, a

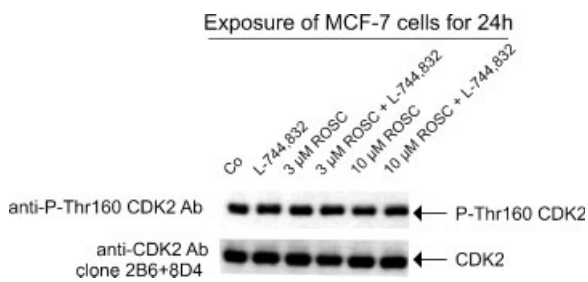

Fig. 10. ROSC at low concentrations does not inhibit phosphorylation of CDK2. Exponentially growing MCF-7 cells were treated for 24 h with ROSC alone, or in combination with the FTI, and then lyzed. WCLs were loaded on 15% SDS-gels. Conditions of separation and immunoblotting were described in detail in Figure 2. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

"gain of function" mutation in the *ras* gene is responsible for the cancer-promoting activity of the protein. The mutation leads to a situation where Ras becomes independent of extracellular stimulation and induces sustained growth-promoting signaling. The majority of anti-cancer drugs is not able to induce cell cycle arrest or apoptosis in tumors bearing this kind of mutation. Therefore, one prime target in treating cancers with mutated Ras was to prevent the post-translational modifications of the protein, thereby rendering it incapable of binding to the proximal side of the plasma membrane what in turn abolishes activation of the GTP-hydrolyzing properties of the protein [Gibbs et al., 1994; Prendergast, 2000]. One such drug is the FTI L-744,832 that prevents the farnesylation of the c-Ha-Ras protein, thereby inhibiting its growth-promoting and anti-apoptotic activities.

To enhance the effect of chemotherapeutic intervention, a combination of two or more drugs targeting distinct cellular pathways is often an advantage. One potential drug to be combined with FTIs, ROSC, is a novel anti-cancer drug that is applied in the treatment of hematological malignancies such as multiple myeloma and B-cell leukemia [Fischer and Gianella-Borradori, 2005]. These clonal tumors are characterized by disruption of the proper balance between pro- and anti-apoptotic factors. There is also a rationale for potential applications in breast and ovarian cancers, especially, those with mutations in the *BRCA1* gene or deficiency in the pro-apoptotic factors. Human MCF-7 breast cancer cells are known to be resistant to a number of conventional drugs [Wesierska-Gadek et al., 2003]. The reduced susceptibility of human MCF-7 cells to agents inducing apoptosis is attributable to a lack of caspase-3 expression due to a 47bp deletion within exon 3 of the *caspase-3* gene [Janicke et al., 1998]. ROSC is a highly efficient drug towards MCF-7 cells and reduces their proliferation not only by induction of the cell cycle arrest but also by induction of apoptosis [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2006b]. ROSC targets in MCF-7 cells two independent pathways: it strongly inhibits CDKs and additionally activates wt p53 protein [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2006b]. The activation of wt p53 protein is mediated by its site-specific phosphorylation and is essential for the induction of apoptosis. The tumor suppressor protein p53, phosphorylated at serine 46, induces the transcription of p53AIP1 protein which is involved in the dissipation of the potential of the mitochondrial membrane [Wesierska-Gadek et al., 2005a]. The phosphorylation of p53 protein at serine 46 is catalyzed by HIPK2 [Wesierska-Gadek et al., 2007]. Surprisingly, endogenous HIPK2, unlike ATM kinase, becomes strongly activated after exposure of MCF-7 cells to ROSC [Wesierska-Gadek et al., 2007].

ROSC is not mutagenic and well tolerated by patients [de la Motte and Gianella-Borradori, 2004]. Nevertheless, the dose of ROSC that is required for anti-proliferative activity is for some tumor cells quite high and, therefore, carboxylate, a ROSC metabolite [de la Motte and Gianella-Borradori, 2004], can reach high concentrations in urine. The exposure of the renal tubuli to increased carboxylate concentrations may cause adverse side-effects [de la Motte and Gianella-Borradori, 2004]. Therefore, an optimized treatment that leads to a significant decrease in the amount of necessary ROSC, can be reached by combination therapies with other drugs or with radiation. In the present study, we show such an approach to combine two drugs: ROSC and L-744,832. Most importantly, FTIs have demonstrated additive or synergistic effects when used in combination with other drugs [Moasser et al., 1998; Edamatsu et al., 2000; Smalley and Eisen, 2003] or radiotherapy [Delmas et al., 2002], in terms of cell cycle arrest and pro-apoptotic activity. In some of these cases, a resistance of the cancer cells could even be counteracted with the combined therapy [Delmas et al., 2002; Smalley and Eisen, 2003]. Although the results are encouraging, the detailed mechanistical background for the observed effects still remains to be elucidated. Possibly, also the observed inhibition of farnesylation of proteins other than Ras could contribute to the unexpected effect of FTIs in some of the studies [Fokstuen et al., 1997].

Our results clearly indicate that this strategy works as expected in vitro and suggests to confirm the findings in an in vivo setting. Importantly, the best results were obtained after treating the cells with a combination of ROSC and L-744,832 for 24 h, and then changing to an incubation of the cells with L-744,832 alone. When applying this schedule, the ratio of living cells decreased to a value

that was significantly lower as compared to a combined treatment together with L-744,832 and ROSC followed by a drug-free post-incubation period. Importantly, a significantly lower concentration of ROSC was needed to achieve the same effect. A lower ROSC concentration should strongly diminish adverse side-effects originating from high-dose ROSC treatment in the clinical situation. As suggested by our in vitro model, when setting up the regimen for the in vivo treatment, one should consider the finding that the effect of the FTI is not immediate but that the drug exerts its biological capacity only after a lag phase of more than 1 day, presumably due to the long turn-over of the modified Ras protein.

Activated Ras has a number of downstream effectors. The best characterized and most investigated targets are the Raf kinase [Chong et al., 2003] and phosphatidylinositol 3′-kinases (PI3K) [Cantley, 2002]. Ras activates Raf kinase that in turn promotes the induction of a cascade resulting in the activation of ERK1/2 mitogen activated protein kinases. The activation of PI3K leads to accumulation of phosphatidylinositol 3′4′5′-triphosphate and in consequence to activation of AKT kinase. However, it should be mentioned that the outcome of activated Ras largely depends on the status of distinct tumor suppressor proteins and therefore, differs between distinct cancer cells and is not always easily predictable.

Interestingly, we found that L-744,832 enhanced the ROSC-mediated activation of the tumor suppressor protein p53. The induction of p53 negatively regulates the cell cycle and apoptosis but it could additionally affect the Ras-signaling pathway. It has for instance been reported that p53 inhibits Net, an effector of Ras/extracellular signal-regulated kinase signaling [Nakade et al., 2004].

A recent publication [Cohen-Jonathan et al., 2001] indicated that L-744,832 sensitizes cancer cells in vivo to radiation therapy by reducing hypoxia in the tumor tissue. This is highly interesting, considering the finding, that hypoxia protects tumor cells from radiation as well as from chemotherapy [Koch et al., 2003; Song et al., 2006] and therefore, FTIs could have an even stronger effect in vivo by reducing hypoxia in cancer cells. Furthermore, L-744,832 was shown to prevent the upregulation of TGF-alpha (transforming growth factor-alpha), a ligand of EGFR (epidermal growth factor receptor), that is mediated by over-expressed c-Ha-Ras in epithelial cells [Sizemore et al., 1999]. A very recent article also showed that while farnesylation is necessary for c-Ha-Ras to function, it is not a prerequisite for the signal transduction of wild-type Ras in non-neoplastic cells [Khwaja et al., 2006]. This difference between c-Ha-Ras and wild-type Ras could also explain why the toxicity of FTIs towards wild-type cells is quite low.

In the present study, we show evidence that the FTI L-744,832 possesses the promising property of sensitizing human breast cancer cells to the growth-suppressive and pro-apoptotic action of the well-established chemotherapeutic drug and CDK inhibitor, ROSC. Importantly, a schedule of combined treatment followed by post-incubation with the FTI alone, revealed the strongest effect and should make it possible to clearly decrease the dose of ROSC, thereby minimizing the adverse side-effects of chemotherapy. Although further research will be needed, this should be a strong argument to support efforts to replace high-dose ROSC treatment with a treatment regimen that employs a combination of ROSC with L-744,832 for the optimal benefit of the patient in the clinical situation. Taken together, the most salient finding of the presented work is that the optimized effect of both drugs achieved via combined therapy with ROSC and the FTI for 1 day and subsequent post-treatment with L-744,832 alone, should open a much wider therapeutic window that results in a clear benefit for the patients.

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REFERENCES

Bargonetti J, Manfredi JJ. 2002. Multiple roles of the tumor suppressor p53. Curr Opin Oncol 14:86–91.

Blagosklonny MV. 2002. P53: An ubiquitous target of anticancer drugs. Int J Cancer 98:161–166.

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. Science 296:1655–1657.

Chong H, Vikis HG, Guan KL. 2003. Mechanisms of regulating the Raf kinase family. Cell Signal 15:463–469.

Cohen-Jonathan E, Evans SM, Koch CJ, Muschel RJ, McKenna WG, Wu J, Bernhard EJ. 2001. The farnesyltransferase inhibitor L744,832 reduces hypoxia in tumors expressing activated H-ras. Cancer Res 61: 2289–2293.

David-Pfeuty T. 1999. Potent inhibitors of cyclin-dependent kinase 2 induce nuclear accumulation of wild-type p53 and nucleolar fragmentation in human untransformed and tumor-derived cells. Oncogene 18:7409–7422.

De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. 1997. Inhibition of cyclin-dependent kinases by purine analogues: Crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem 243:518–526.

de la Motte S, Gianella-Borradori A. 2004. Pharmacokinetic model of R-roscovitine and its metabolite in healthy male subjects. Int J Clin Pharmacol Ther 42:232–239.

Delmas C, Heliez C, Cohen-Jonathan E, End D, Bonnet J, Favre G, Toulas C. 2002. Farnesyltransferase inhibitor, R115777, reverses the resistance of human glioma cell lines to ionizing radiation. Int J Cancer 100:43–48.

Eckert LB, Repasky GA, Ulku AS, McFall A, Zhou H, Sartor CI, Der CJ. 2004. Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. Cancer Res 64:4585–4592.

Edamatsu H, Gau CL, Nemoto T, Guo L, Tamanoi F. 2000. Cdk inhibitors, roscovitine and olomoucine, synergize with farnesyltransferase inhibitor (FTI) to induce efficient apoptosis of human cancer cell lines. Oncogene 19: 3059–3068.

Fischer PM, Gianella-Borradori A. 2005. Recent progress in the discovery and development of cyclin-dependent kinase inhibitors. Expert Opin Invest Drugs 14:457–477.

Fokstuen T, Rabo YB, Zhou JN, Karlson J, Platz A, Shoshan MC, Hansson J, Linder S. 1997. The Ras farnesylation inhibitor BZA-5B increases the resistance to cisplatin in a human melanoma cell line. Anticancer Res 17:2347–2352.

Gibbs JB, Oliff A, Kohl NE. 1994. Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. Cell 77:175–178.

Gray N, Detivaud L, Doerig C, Meijer L. 1999. ATP-site directed inhibitors of cyclin-dependent kinases. Curr Med Chem 6:859–875.

Jacks T, Weinberg RA. 1998. The expanding role of cell cycle regulators. Science 280:1035–1036.

Janicke RU, Sprengart ML, Wati MR, Porter AG. 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273:9357–9360.

Khwaja A, Dockrell ME, Hendry BM, Sharpe CC. 2006. Prenylation is not necessary for endogenous Ras activation in non-malignant cells. J Cell Biochem 97:412–422.

Koch S, Mayer F, Honecker F, Schittenhelm M, Bokemeyer C. 2003. Efficacy of cytotoxic agents used in the treatment of testicular germ cell tumours under normoxic and hypoxic conditions in vitro. Br J Cancer 89: 2133–2139.

Kotala V, Uldrijan S, Horky M, Trbusek M, Strnad M, Vojtesek B. 2001. Potent induction of wild-type p53-dependent transcription in tumour cells by a synthetic inhibitor of cyclin-dependent kinases. Cell Mol Life Sci 58:1333–1339.

Ljungman M, Paulsen MT. 2001. The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382. Mol Pharmacol 60:785–789.

Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP. 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 243:527–536.

Moasser MM, Sepp-Lorenzino L, Kohl NE, Oliff A, Balog A, Su DS, Danishefsky SJ, Rosen N. 1998. Farnesyl transferase inhibitors cause enhanced mitotic sensitivity to taxol and epothilones. Proc Natl Acad Sci U S A 95: 1369–1374.

Motokura T, Arnold A. 1993. Cyclins and oncogenesis. Biochim Biophys Acta 1155:63–78.

Nakade K, Zheng H, Ganguli G, Buchwalter G, Gross C, Wasylyk B. 2004. The tumor suppressor p53 inhibits Net, an effector of Ras/extracellular signal-regulated kinase signaling. Mol Cell Biol 24:1132–1142.

North S, Pluquet O, Maurici D, El-Ghissassi F, Hainaut P. 2002. Restoration of wild-type conformation and activity of a temperature-sensitive mutant of p53 (p53(V272M)) by the cytoprotective aminothiol WR1065 in the esophageal cancer cell line TE-1. Mol Carcinog 33:181–188.

Nurse P. 1997. Reductionism. The ends of understanding. Nature 387:657.

Nurse P. 2002. Cyclin dependent kinases and cell cycle control (nobel lecture). Chembiochem 3:596–603.

Prendergast GC. 2000. Farnesyltransferase inhibitors: Antineoplastic mechanism and clinical prospects. Curr Opin Cell Biol 12:166–173.

Prives C, Hall PA. 1999. The p53 pathway. J Pathol 187: 112–126.

Rodriguez-Puebla ML, Robles AI, Conti CJ. 1999. ras activity and cyclin D1 expression: An essential mechanism of mouse skin tumor development. Mol Carcinog 24: 1–6.

Senderowicz AM. 2003. Small-molecule cyclin-dependent kinase modulators. Oncogene 22:6609–6620.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512.

Sizemore N, Cox AD, Barnard JA, Oldham SM, Reynolds ER, Der CJ, Coffey RJ. 1999. Pharmacological inhibition of Ras-transformed epithelial cell growth is linked to down-regulation of epidermal growth factor-related peptides. Gastroenterology 117:567–576.

Smalley KS, Eisen TG. 2003. Farnesyl transferase inhibitor SCH66336 is cytostatic, pro-apoptotic and enhances chemosensitivity to cisplatin in melanoma cells. Int J Cancer 105:165–175.

Song X, Liu X, Chi W, Liu Y, Wei L, Wang X, Yu J. 2006. Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1alpha gene. Cancer Chemother Pharmacol 58:776–784.

Toledo F, Wahl GM. 2006. Regulating the p53 pathway: In vitro hypotheses, in vivo veritas. Nat Rev Cancer 6:909–923.

van den Heuvel S, Harlow E. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262: 2050–2054.

Vindelov LL, Christensen IJ, Nissen NI. 1983. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323–327.

Vogelstein B, Kinzler KW. 2004. Cancer genes and the pathways they control. Nat Med 10:789–799.

Weinberg RA. 1996. The molecular basis of carcinogenesis: Understanding the cell cycle clock. Cytokines Mol Ther 2:105–110.

Wesierska-Gadek J. 2005. Major contribution of the multidrug transporter P-glycoprotein to reduced susceptibility of poly(ADP-ribose) polymerase-1 knock-out cells to doxorubicin action. J Cell Biochem 95:1012–1028.

Wesierska-Gadek J, Schmid G. 2000. Overexpressed poly-(ADP-ribose) polymerase delays the release of rat cells from p53-mediated G(1) checkpoint. J Cell Biochem 80: 85–103.

Wesierska-Gadek J, Hohenauer H, Hitchman E, Penner E. 1995. Autoantibodies from patients with primary biliary cirrhosis preferentially react with the amino-terminal domain of nuclear pore complex glycoprotein gp210. J Exp Med 182:1159–1162.

Wesierska-Gadek J, Bugajska-Schretter A, Cerni C. 1996. ADP-ribosylation of p53 tumor suppressor protein: Mutant but not wild-type p53 is modified. J Cell Biochem 62:90–101.

Wesierska-Gadek J, Schloffer D, Kotala V, Horky M. 2002. Escape of p53 protein from E6-mediated degradation in HeLa cells after cisplatin therapy. Int J Cancer 101:128–136.

Wesierska-Gadek J, Gueorguieva M, Horky M. 2003. Dual action of cyclin-dependent kinase inhibitors: Induction of cell cycle arrest and apoptosis. A comparison of the effects exerted by roscovitine and cisplatin. Pol J Pharmacol 55:895–902.

Wesierska-Gadek J, Gueorguieva M, Horky M. 2005a. Roscovitine-induced up-regulation of p53AIP1 protein precedes the onset of apoptosis in human MCF-7 breast cancer cells. Mol Cancer Ther 4:113–124.

Wesierska-Gadek J, Gueorguieva M, Ranftler C, Zerza-Schnitzhofer G. 2005b. A new multiplex assay allowing simultaneous detection of the inhibition of cell proliferation and induction of cell death. J Cell Biochem 96: 1–7.

Wesierska-Gadek J, Kramer M, Schmid G. 2006a. Prevention of farnesylation of c-Ha-Ras protein enhances synergistically the cytotoxic action of doxorubicin in cycling but not in quiescent cells. J Cell Biochem 99: 1664–1676.

Wesierska-Gadek J, Schreiner T, Gueorguieva M, Ranftler C. 2006b. Phenol red reduces ROSC mediated cell cycle arrest and apoptosis in human MCF-7 cells. J Cell Biochem 98:1367–1379.

Wesierska-Gadek J, Schmitz C-L, Ranftler C. 2007. Roscovitine-activated HIP2 kinase induces phosphorylation of wt p53 at Ser-46 in human MCF-7 breast cancer cells. J Cell Biochem in press

Wojciechowski J, Horky M, Gueorguieva M, Wesierska-Gadek J. 2003. Rapid onset of nucleolar disintegration preceding cell cycle arrest in roscovitine-induced apoptosis of human MCF-7 breast cancer cells. Int J Cancer 106:486–495.