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Prevention of Farnesylation of c-Ha-Ras Protein Enhances Synergistically the Cytotoxic Action of Doxorubicin in Cycling but not in Quiescent Cells

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Abstract Ras, the product of a proto-oncogene, is a GTP-hydrolyzing enzyme found mutated in approximately 50% of human cancers. ''Gain of function'' mutations of Ras lead to an escape of transformed cells from cell-cycle control, rendering them independent to stimulation by growth factors, giving them almost unlimited proliferation capacity. The cytosolic precursor isoform of Ras is biologically inactive. After several post-translational modifications, Ras is anchored to the plasma membrane and, thereby, the protein becomes activated. The finding that lipid modifications of Ras protein, particularly farnesylation, are essential for its signal transduction activity, gave rise to the concept that blocking farnesyl protein transferase (FPTase), the enzyme catalyzing the first step in the Ras modification cascade, would prevent proper membrane anchoring and provide an improved approach in the cure of tumors harboring Ras mutations. In the present study we used transformed rat cells overexpressing a temperature-sensitive p53 protein, adopting wt conformation at 32°C and mutant conformation at 37°C. We treated the cells growing at 32 or 37°C with doxorubicin alone, or in combination with inhibitors of FPTase. Combined treatment was more efficient and the same inhibition of cell proliferation was reached at lower DOX concentrations. The treatment strongly affected the growth rate of tumor cells but only negligibly of normal cells. However, the inhibitors of FPTase prevented the membrane anchoring in both situations. These results show two striking advantages of the combined treatment: the desired cytostatic effect on tumor cells at lower drug concentrations and clearly reduced adverse effects on quiescent cells. J. Cell. Biochem. 99: 1664–1676, 2006.

Key words: p53 tumor suppressor; cell-cycle arrest; signal transduction; c-Ha-Ras localization; small GTP-binding proteins; GTPase; isoprenylation

In many cancers mutation of the *ras* gene is an early event in the onset of neoplastic transformation. The Ras proteins are, in their activated forms, located on the proximal side of the plasma membrane and are gate-keepers for one of the major pathways mediating growth factor stimulation. Ras is activated by exchange of bound guanosine 5′-diphosphate (GDP) for guanosine 5′-triphosphate (GTP). This exchange is stimulated by extracellular signals, including activation of receptor tyrosine kinases by their corresponding growth factors. Among the targets of Ras are Raf-1 and phosphatidyl-inositol 3-kinase (PI3-kinase). Activated Raf-1 initiates a phosphorylation cascade that ultimately leads to the activation of transcription factors. Induction of PI3-kinase mediates suppression of apoptosis, enhanced cell motility, and increased invasiveness of tumors [Bishop et al., 2003]. In humans

Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; DOX, doxorubicin; FPTase, farnesyl protein transferase; FTI, farnesyl protein transferase inhibitor; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; MDM-2, mouse double minute-2; MCM7, minichromosome maintenance 7; PCNA, proliferating cell nuclear antigen; PD, Petri dish; PVDF, polyvinylidene difluoride; Ran, Ras-related nuclear; RBD, Raf binding domain; REFs, rat embryo fibroblasts; ts, temperature-sensitive: WCL, whole cell lysate; WT, wild-type.

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three different *ras* genes code for four isoforms of Ras, namely: Ha-Ras (Harvey), N-Ras (neural), Ki-Ras4A, and Ki-Ras4B (Kirsten) [Bos, 1989]. In human cancers, mutated Ras isoforms are frequently observed, also depending on the type of cancer. When the "gain of function" Ras proteins adopt mutant conformation they sustain their growth-promoting signaling irrespectively of extracellular stimulation, rendering the cell independent of growth factor activation.

Farnesyl protein transferase (FPTase) is a zinc-metalloenzyme that catalyzes the transfer of a farnesyl moiety to the thiol group of the cysteine in the C-terminal CAAX motif of the Ras protein. After farnesylation, Ras has to be proteolytically processed and then the farnesylated cysteine residue within the C-terminal CAAX motif has to be carboxy-methylated and, finally, palmitylated at the adjacent cysteine residue to obtain the functional protein. These post-translational modifications are a prerequisite for membrane anchoring of Ras, which in turn converts the enzyme from its inactive into its active, Ras-GTP bound form. Therefore, one attractive target for an anti-cancer treatment involving Ras would be the prevention of post-translational modifications and membrane-anchoring of the protein. FPTase inhibitors (FTIs) suggest themselves as tools to reach this goal. Interestingly, it was found that inhibition of farnesylation by specific FTIs indeed abrogates the binding of Ras to the plasma membrane and thereby, they are capable of reverting the transformed phenotype of cancer cells [Lerner et al., 1995a]. The exact mechanism by which FTIs exert their inhibiting action on Ras still remains to be elucidated but it was shown that they also prevent the farnesylation of RhoB, other Ras GTPases and of centromere-associated proteins [Ashar et al., 2000; Bishop et al., 2003]. It was also found that Ki-Ras and N-Ras are less affected by FTIs because these two isoforms can alternatively attach to the plasma membrane via geranyl-geranylation [Lerner et al., 1995b; Fiordalisi et al., 2003], a modification performed by the FPTase related enzyme, geranyl-geranyl transferase-I (GGT-I) [James et al., 1996; Rowell et al., 1997; Whyte et al., 1997]. Some FTIs are already in clinical trials although the high expectations could only partly be met [Caponigro et al., 2003; Doll et al., 2004; Appels et al., 2005; Margaritora et al., 2005].

The p53 tumor suppressor protein is a central protein regulating DNA-damage response, cell-cycle progression, and apoptosis [Prives and Hall, 1999; Bargonetti and Manfredi, 2002; Blagosklonny, 2002]. The localization of the protein changes throughout the cell cycle, being mostly nuclear during the G_1 phase of the cell cycle and predominantly cytoplasmic in the S and G_2 phases in normal, unstressed cells [Shaulsky et al., 1990; David-Pfeuty et al., 1996]. The major regulator of p53 protein is MDM-2, an E3 ubiquitin ligase that is itself transcriptionally regulated by p53. Under circumstances under which the cell-cycle inhibiting action of p53 is not required, MDM-2 is able to ubiquitylate p53, thereby marking it for degradation. On the other hand, in response to stress stimuli p53 is stabilized and remains in the nucleus, enabling it to adjust the expression of target genes involved in cell-cycle arrest and apoptosis [Maltzman and Czyzyk, 1984; Fritsche et al., 1993]. In our studies we used a temperature sensitive (ts) mutant of $p53^{135val}$ [Eliyahu et al., 1985] that adopts wild-type (wt) conformation at 32°C and mutant conformation at 37°C. With this mutant it is possible to mimic the situation of p53 mutant and of p53 wt cells in the same genetic background by simply shifting the cells to the corresponding temperature.

The aim of the present work was to examine whether the prevention of the farnesylation of the overexpressed c-Ha-Ras protein would sensitize transformed rat cells to the action of anti-cancer drugs within a dose-range that is non-toxic for quiescent cells. Rat cells expressing a temperature-sensitive p53 mutant [Eliyahu et al., 1985] provided the experimental system to test under the same genetic background the susceptibility of exponentially growing transformed cells versus quiescent cells to doxorubicin (DOX), a frequently used anti-cancer drug, alone or in combination with the inhibitor of FPTase. To ensure that the observed effects are attributable to inhibition of farnesylation, we additionally used inhibitors of carboxy-methylation of Ras such as AFC [Tisch et al., 1996] and FTA [Marom et al., 1995]. Inhibition of protein farnesylation resulted in the loss of the membrane anchoring of the de novo synthesized c-Ha-Ras protein in both proliferating and quiescent cells. On the other hand, the two alternative inhibitors AFC and FTA affected only negligibly the isoprenylation status and the intracellular distribution

of c-Ha-Ras protein. Our results show that the proliferating cells were significantly more sensitive to the action of DOX than the non-dividing cells. Moreover, inhibition of FPTase, but not of protein carboxy-methylation sensitized proliferating cells to the action of DOX. As expected, the desired cytostatic effect on tumor cells had a minimal impact on normal, quiescent cells. This is of high importance for the clinical situation, where patients may benefit from a decreased DOX concentration that will strongly reduce the adverse side-effects of the drug during the treatment.

MATERIAL AND METHODS

Antibodies

Monoclonal anti-p53 antibodies recognizing mutant p53 (PAb240), wt p53 (PAb246), or both (PAb421), and anti-p21/waf-1 antibodies were purchased from Oncogene Research Products (Cambridge, MA). The polyclonal anti-p53 antibody CM-1 was obtained from Novocastra Laboratories Ltd. (Newcastle upon Tyne, England). Anti-MDM-2 (SMG-14) and anti-p21-ras antibodies were from DAKO A/S (Glostrup, Denmark). Monoclonal anti-actin antibodies were from ICN (ICN Biomedicals, Aurora, OH). Distinct secondary antibodies were obtained from Amersham International (Little Chalfont, Buckinghamshire, England).

Plasmids

pLTRp53cGval135 comprising a chimera of mouse p53 cDNA and genomic DNA (generous gift of Dr. M. Oren) has been previously referred to as pLTRp53cG [Eliyahu et al., 1985]. It encodes a mutant protein harboring a substitution from alanine to valine at the amino acid in position 135. The plasmids pVV2, bearing the neomycin resistance sequence, and pVEJB coding for a mutated human c-Ha-Ras gene cloned into pVVJ were used. The Ewing sarcoma cell line overexpressing human ts mutant $p53^{138val}$ was a kind gift of Dr. H. Kovar.

Cell Clones

The establishment of transformed rat cell clones was performed according to a protocol previously described in detail [Wesierska-Gadek et al., 1996] using primary Fisher rat embryo cells. Briefly, 15.5 gestation days old embryos were dissociated into single cell suspension by fractionated trypsinization and plated in DMEM supplemented with 10% FCS in an atmosphere of 7.5% CO_2 to obtain cell layers of 50% confluence the next day. Cells were transfected with the pVV2 plasmid bearing the neomycin resistance sequence. Medium was changed and after 4 h, Ca++-precipitated DNA was added (6 μg DNA per Petri dish (PD) of 6 cm diameter). After 24 h cells were replated at 3×10^5 cells per PD. Geneticin (G418, Life Technologies, Inc., Gaithersburg, MD) was added 3 days later to a final concentration of 200 μg/ml. The selective medium was changed twice a week. G418 resistant colonies were isolated by means of steel cylinders and further propagated in selective medium at a 1:8 transfer schedule. Clones were considered established after 25 population doublings of the founder cell. Cells were grown in DMEM supplemented with 10% FCS in an atmosphere of 7.5% CO_2. For experiments dealing with a change of the conformational state of p53 protein, cells grown at 37°C were shifted to 32°C for indicated periods of time.

Drugs

We used three different agents preventing modifications of Ras proteins from Alexis Biochemicals (Lausen, Switzerland). N-acetyl-S-farnesyl-cysteine (AFC), farnesylthioacetic acid (FTA), and the farnesyltransferase inhibitor [N-[2(S)-[2-(R)-Amino-3-mercapthopropylamino]-3-methylbutyl]-Phe-Met-OH] (FTI). Stock solutions of AFC and FTA were prepared in DMSO and a stock of FTI was diluted in an aqueous solution containing 1% DMSO. Stock solutions were stored until use at −20°C. Doxorubicin (Calbiochem, San Diego, CA) was used in concentrations ranging from 0.05 to 5 μM for indicated periods of time.

Cell Treatment

Cells were cultivated at a basal temperature of 37°C. Then they were shifted to 32°C and kept there for at least 24 h to allow p53 to adopt wt conformation. Thereafter, the cells were treated with different compounds, as indicated, or were alternatively shifted back to 37°C and subjected to the treatment with allocated drugs.

Determination of the Number of Viable Cells

The proliferation of transformed rat cells maintained at 37°C or at 32°C and their

sensitivity to increasing concentrations of DOX were determined by the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). As described recently in more detail [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2004, 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 quadruplicate. Luminescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter. Each point represents the mean ± SEM (bars) of replicates from one representative experiment. At least four independent experiments were performed; each individual sample was measured in quadruplicate. Statistical significance of the results was evaluated using Bonferroni's Multiple comparison test.

Measurement of the DNA Content of Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis based on a slightly modified method [Wesierska-Gadek and Schmid, 2000] described, previously [Vindelov et al., 1983]. The cells were detached from substratum by trypsinization, and then all cells were harvested by centrifugation and washed in PBS. Aliquots of 1×10^6 cells were used for further analysis. Cells were stained with propidium iodide as described, previously [Wesierska-Gadek and Schmid, 2001]. Fluorescence was measured using the Becton Dickinson FACScan after at least 2 h incubation of the cells at +4°C in the dark.

Pull-Down Assay

The pull-down assay was performed essentially as described earlier [de Rooij and Bos, 1997]. GST-tagged recombinant protein encompassing the Ras binding domain of Raf-1 (Raf/RBD) was purified by adsorption to glutathione GST-Sepharose beads (Pharmacia) and used for detection of activated Ras protein in cell lysates. Untreated control cells and cells treated with FTI were lyzed in RIPA buffer supplemented with protease inhibitors. Cell lysates were cleared by centrifugation at 15,000*g* for 5 min at 4°C. The supernatant (100 μg) was loaded on Raf/RBD immobilized on GST-Sepharose beads for 20 min. Samples were washed four times with RIPA buffer, eluted with sample buffer and loaded on 15% SDS gels. Ras protein was visualized by immunoblotting using a monoclonal anti-c-Ha-Ras antibody.

Cell Fractionation

Cells were harvested, washed with PBS and resuspended in ice-cold hypotonic buffer supplemented with protease inhibitors [Wesierska-Gadek et al., 2005a]. After addition of Nonidet-P-40 and Na-deoxycholate to a final concentration of 1 and 0.5%, respectively, cells were vigorously vortexed and centrifuged at 15,000*g* for 15 min at +4°C. Supernatant defined as cytosolic fraction and residual insoluble fraction defined as membrane-bound (MB) fraction were analyzed separately.

Immunoblotting

Proteins were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane. Immunodetection of antigens was performed with specific antibodies and enhanced chemiluminescent detection reagent ECL (Amersham International). Equal loading of proteins was confirmed by Ponceau S staining and additionally by sequential incubation of blots with anti-actin or with anti-ran antibodies [Wesierska-Gadek et al., 1995]. Ran–Ras-related nuclear protein ($M_{wt} = 26$ kDa) is a small GTPase of the Ras superfamily.

RESULTS

Cell-Cycle Arrest Induced by wt p53 Protein

In the first step the features of the cell clones of c-Ha-Ras-transformed rat cells, additionally expressing a temperature sensitive $p53^{135val}$ mutant, were examined. The overexpression of c-Ha-Ras and $p53^{135val}$ in REFs dramatically changed the phenotype of the cells (Fig. 1, upper panel). Generated cell clones exhibited features characteristic for highly transformed cells. These cells show a spindle-like shape and lamellipodia due to changes in the cytoskeletal organization. The transformation induced by Ras in cooperation with other proteins (e.g., TGF-β) is also referred to as epithelial mesenchymal transition (EMT) [Helftenbein et al., 1993; Zondag et al., 2000; Janda et al., 2002].

Proliferation of cells was examined by a traditional procedure based on cell counting and additionally through an output method based on the determination of the cellular level of ATP. At basal temperature, at which

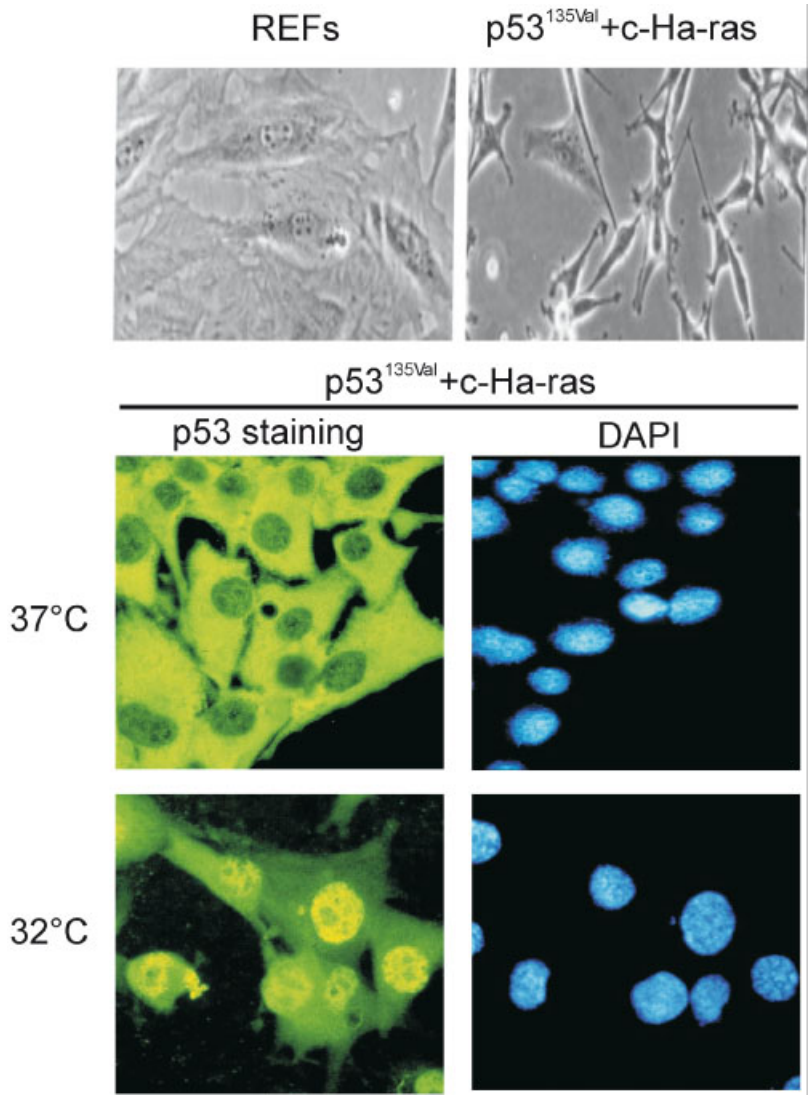

Fig. 1. Switch from mutant to wt p53 conformation is accompanied by p53 translocation from the cytoplasm to the nucleus. **Upper panel**: Phenotype of rat embryo fibroblasts (REFs) and $p53^{135val}$ + c-Ha-Ras transformed rat cells. Control REFs and cells transformed with $p53^{135val}$ + c-Ha-Ras were examined under phase contrast microscopy. **Lower panel**: Intracellular localization of ts $p53^{135val}$ mutant in transformed rat cells depends on its phenotype. Transformed cells cultivated at 37°C or at 32°C were fixed and stained with monoclonal anti-p53 antibody PAb421. The nuclei were visualized with DAPI. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

p53 exhibited its mutant character and was transcriptionally inactive, cells proliferated very strongly. After temperature shift from 37 to 32°C cell proliferation was inhibited (Fig. 2A). Interestingly, a difference occurred between results obtained by both methods during the time window between 24 and 48 h. While the cell numbers remained almost unchanged, the ATP level increased approximately fourfold. It seems that the block of cell proliferation and changes of cell shape resulted in the elevation of cellular ATP levels. After 72 h at 32°C the ATP level remained constant and was exactly the same as at 48 h after temperature shift.

The stop of cell proliferation was due to an arrest of the transformed cells in the G_1 phase of the cell cycle. After 24 h at 32°C approximately 90% of cells were captured in G_1 phase (Fig. 2B–D). The cell-cycle arrest was induced by p53 protein present in wt conformation. As confirmed by immunofluorescence staining of cells, after lowering the temperature from 37 to 32°C p53 adopted wt conformation (Fig. 1; lower

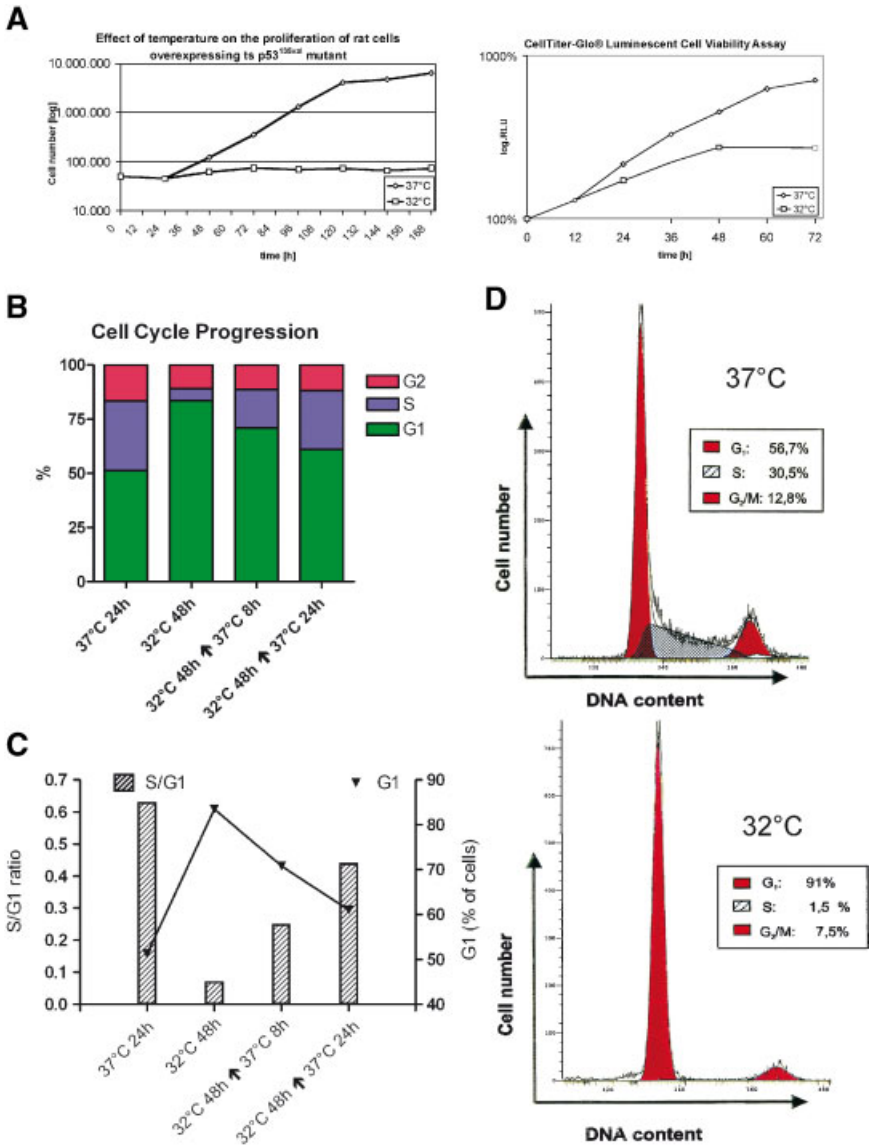

Fig. 2. Switch from mutant to wt conformation of p53 protein results in cell-cycle arrest. **A**: Rapid proliferation of rat cells transformed by $p53^{135val}$ + c-Ha-Ras is inhibited at the restrictive temperature (32°C). Cell numbers were determined by cell counting (**left panel**) and by CellTiterLumiGlo (**right panel**). The latter method is based on the measurement of the concentration of cellular ATP. Each time point represents the mean of 16 replicates ± SD. Values of SD are very low and therefore are not visible. **B**: Distribution of the cells in distinct phases of the cell cycle. **C**: Reversible G_1 cell-cycle block of $p53^{135val}$ + c-Ha-Ras transformed rat cells at the restrictive temperature (32°C). **D**: DNA histogramme depicting the typical DNA profile of $p53^{135val}$ + c-Ha-Ras transformed rat cells at 37°C and at 32°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

panel) and translocated from the cytosol into the nucleus where it could execute its anti-proliferative functions (Fig. 1; lower panel). In this environment p53 regained its impact as transcription factor and upregulated its target genes, for example, MDM-2 (Fig. 3, upper panel) leading to the observed effect on the cell-cycle regulation. It is noteworthy to mention that wt p53 did not affect the cellular level of c-Ha-Ras protein (Fig. 3, lower panel).

Mutated Ras Protein is Biologically Active

To assess the activity of c-Ha-Ras protein in transformed rat cells, the pull-down assay was performed. This assay is based on the fact that only activated, that is, GTP-loaded Ras is capable to bind the Ras binding domain of Raf-1 (Raf/RBD). This binding capacity of the activated protein can be used to perform a highly selective purification. The precleared cell

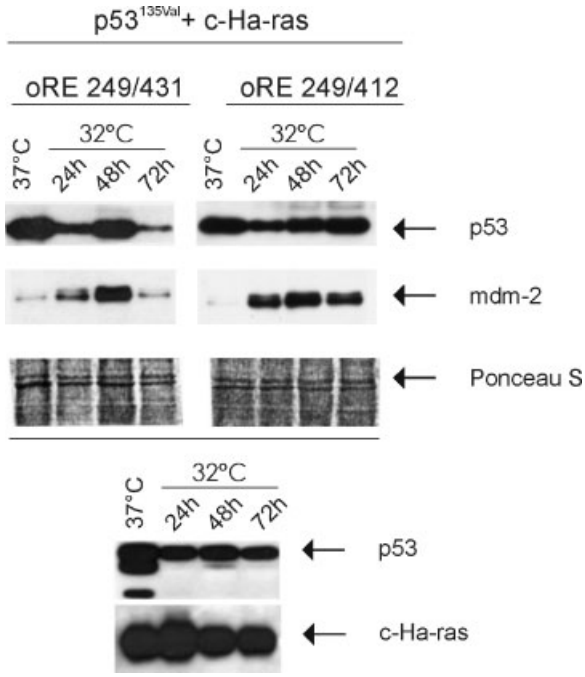

Fig. 3. ts $p53^{135val}$ protein acts as tumor suppressor at 32°C. **Upper panel**: Two different cell clones were cultivated at 37°C and then shifted to 32°C for 24, 48, or 72 h. Cells were lyzed and WCLs were analyzed by immunoblotting on 10% SDS gels. The membrane was incubated with the anti-p53 antibody PAb421 and sequentially with an anti-MDM-2 antibody (SMP14). Control of loading was done with anti-actin antibodies and Ponceau S staining. **Lower panel**: WCLs were separated on 15% SDS gels to detect c-Ha-Ras.

lysates prepared from cells cultivated at basal temperature were loaded on RBD immobilized on GST-Sepharose. As shown in Figure 4, Ras was pulled down from the RBD-Sepharose beads.

Inhibitors of Isoprenylation Are not Cytotoxic

To avoid any undesired unspecific effects exerted by the tested inhibitors, their cytotoxic potential was examined. Dividing as well as G_1 arrested rat cells transformed with c-Ha-Ras and $p53^{135val}$ were exposed for 24 h to the three different agents. Thereafter, the number of viable cells was determined. As shown in Figure 5A, the tested inhibitors exhibited no direct cytotoxicity and did not induce apoptosis.

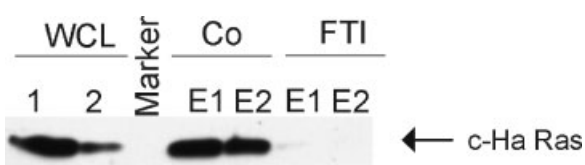

Fig. 4. Unprenylated c-Ha-Ras lost its biological activity. WCLs prepared from control cells or cells exposed to FTI for 24 h were loaded on beads on which Raf-RBD recombinant protein was immobilized. After washing, the bound proteins were pulled down. The elution procedure was performed twice (eluates E1 and E2). WCLs were loaded as a control in a high (**lane 1**) and a low (**lane 2**) concentration.

Prevention of Membrane-Anchoring of c-Ha-Ras Protein Abolishes the Biological Activity of c-Ha-Ras

In the next step we tested three compounds for their ability to prevent the proper post-translational modifications of the c-Ha-Ras protein and its association with the plasma membrane. Whole cell lysates (WCLs) and subcellular fractions obtained from control and cells treated with inhibitors of Ras processing were resolved on SDS-gels and analyzed by immunoblotting. As depicted in Figure 5B, in untreated control cells c-Ha-Ras protein was completely anchored to the membrane and no free Ras protein could be detected. However, exposure of cells to FTI, a specific inhibitor of FPTase, rendered c-Ha-Ras soluble and a substantial amount of the c-Ha-Ras protein accumulated in the cytosolic fraction. Interestingly, two alternative inhibitors of Ras modification, FTA and AFC were less effective. Only a small proportion of c-Ha-Ras became soluble after treatment with AFC. The cell-cycle status of treated cells was not essential for the efficacy of the inhibitors of FPTase. FTI prevented membrane anchoring of c-Ha-Ras in cycling as well as in G_1 arrested cells (Fig. 5C). Moreover, the prevention of the membrane anchoring of c-Ha-Ras protein abolished its biological activity as evidenced by the pull-down assay (Fig. 4). Blots were sequentially incubated with anti-ran antibodies to prove the protein loading. Ran is a very abundant small GTPase of the Ras superfamily that controls nucleocytoplasmic transport and therefore shuttles between cytoplasm and nucleus. Therefore, detection of ran protein is suitable to check the loading of proteins originating from distinct subcellular compartments.

No Effect of Inhibition of FPTase on the Intracellular Localization of the Endogenous Ras Protein in Primary Rat Cells

To assess the effect of the treatment of normal rat fibroblasts with inhibitors of FPTase on the endogenous Ras protein, the untreated and the FTI-treated cells were fractionated and the distribution of endogenous Ras protein was analyzed by immunoblotting. As shown in

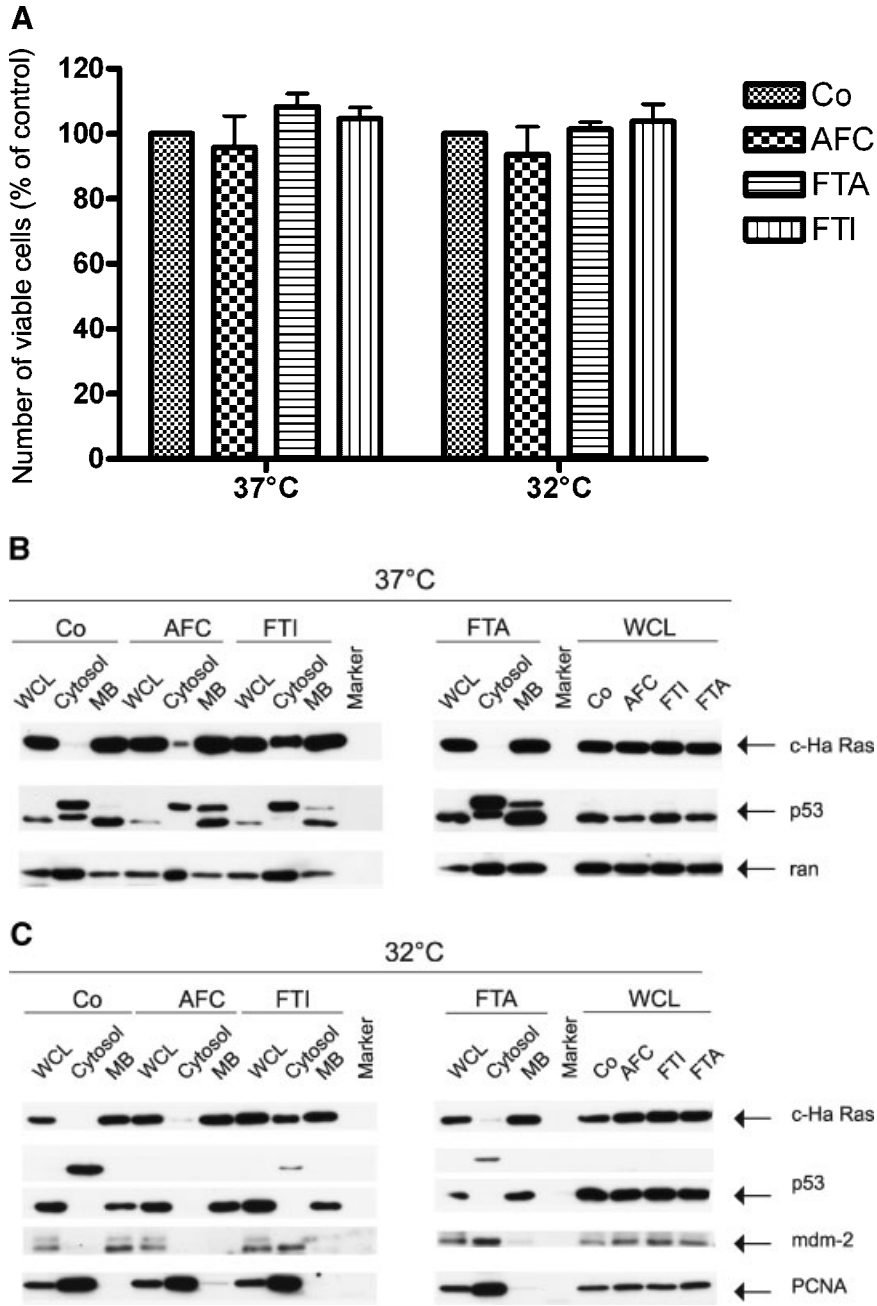

Fig. 5. Inhibition of FPTase by the specific inhibitor FTI reduces the anchoring of c-Ha-Ras at the plasma membrane. **A**: Tested inhibitors are not cytotoxic. Rat cells were exposed to AFC (20 μM), FTA (20 μM), or to FTI (50 μM) for 24 h. The number of viable cells was determined by CellTiterLumiGlo and normalized against the cell number in controls. **B**: Control cells and cells exposed to inhibitors for 24 h at 37°C were fractionated. WCL, soluble fraction (cytosol) and insoluble pellet (membrane-bound fraction) were analyzed by immunoblotting. **C**: Control cells and cells exposed to inhibitors for 24 h at 32°C were fractionated. WCL, soluble fraction (cytosol) and insoluble pellet (membrane-bound fraction) were analyzed by immunoblotting.

Figure 6, primary rat cells expressed Ras protein at a very low level. In untreated control cells a minor part of Ras protein was soluble and was therefore detected in the cytosolic fraction. Exposure of primary rat cells to FTI for 24 h did not result in the accumulation of soluble Ras protein in the cytosol.

Increased Accumulation of Unprenylated Ras After Longer Inhibition of FPTase

The exposure of transformed rat cells for 24 h to FTI markedly abolished farnesylation of c-Ha-Ras protein (Fig. 5B,C). Interestingly, after longer exposure of transformed rat cells to FTI the

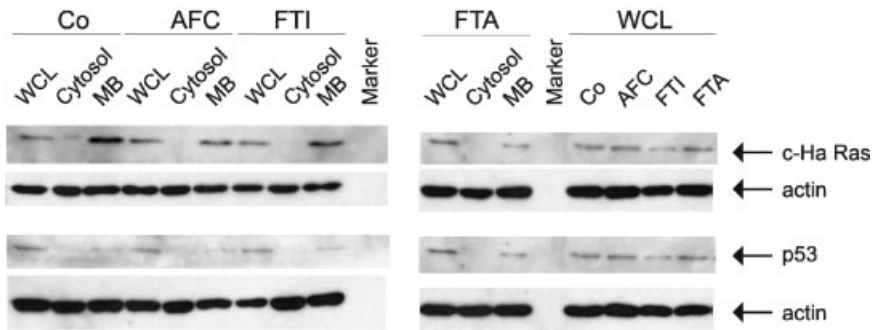

Fig. 6. No effect of FTI on the Ras localization in normal rat fibroblasts. Control REFs and cells exposed to inhibitors for 24 h were fractionated. WCL, soluble fraction (cytosol) and insoluble pellet (membrane-bound fraction) were analyzed by immunoblotting.

amount of unprenylated soluble c-Ha-Ras was markedly increased (data not shown). The analysis of the MB fraction isolated from untreated control cells and cells treated with the FTI for 48 h revealed a marked decrease in the lower protein band representing prenylated and processed c-Ha-Ras protein [Cox et al., 1995] after treatment. Moreover, the upper protein band representing unprenylated c- Ha-Ras protein appeared. How could one explain the association of the unprenylated c-Ha-Ras protein with this fraction? It seems that after inhibition of farnesylation the unmodified c-Ha-Ras protein is at least partially localized in Golgi [Wurzer et al., 2001]. Wurzer et al. [2001] showed by immunofluorescence staining that after inhibition of FPTase c-Ha-Ras protein was partially localized in the Golgi apparatus. Since the fractionation performed in the transformed cells was primarily designed to separate soluble c-Ha-Ras protein from its farnesylated form anchored to the plasma membrane, it is clear that the residual insoluble fraction was heterogeneous and contained constituents of different subcellular compartments.

These results indicate that the turnover of the processed c-Ha-Ras protein is slow and that longer inhibition of FPTase reduces the population of membrane-anchored c-Ha-Ras protein more efficiently. The two isoforms of c-Ha-Ras protein were separated with higher efficiency on SDS-gels prepared with a higher concentration of the cross-linking agent bis-acrylamide (Fig. 7).

DOX Strongly Inhibits Proliferation of Dividing Cells

Under in vivo conditions rapidly proliferating cancer cells in tumors are surrounded by normal, non-neoplastic cells that divide rather slowly. To mimic this situation we performed the following experiments with dividing and G_1 arrested rat cells transformed with c-Ha-Ras and $p53^{135val}$. Cells plated into microtiter plates (two plates for each condition) were exposed to increasing concentrations of DOX for 24 h. Thereafter, the number of viable cells was determined in the first microtiter plate. In the second microtiter plate medium was changed and cells were incubated for a further 48 h in a drug-free medium. The measurement of the number of viable cells immediately after treatment for 24 h delivered information on the direct cytotoxic effect of the drug. On the other hand, post-incubation of cells treated for 24 h, for another 48 h in a drug-free medium, allowed the evaluation of the long-term effects of the treatment. The latter assay is more relevant to the clinical situation. As expected, cycling cells at 37°C were stronger affected by DOX than G_1 arrested cells (Fig. 8). The dividing cells were approximately fivefold more sensitive than

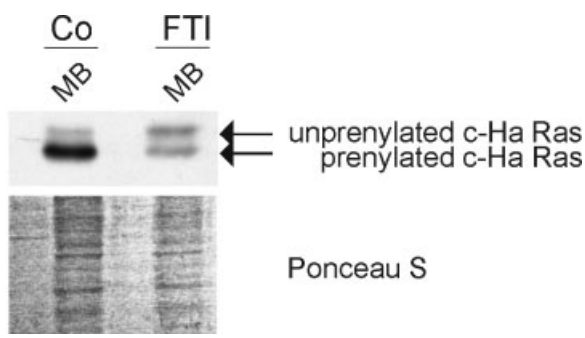

Fig. 7. Decrease of prenylated c-Ha-Ras protein associated with membrane-bound fraction after longer exposure to the inhibitor of FPTase. Membrane-bound fraction prepared from control cells and cells treated with FTI for 48 h were loaded. To better resolve unprenylated and prenylated form of c-Ha-Ras, the higher concentration of the cross-linker was used for gel preparation.

quiescent cells. The difference of the IC_{50} between cells maintained at 37°C and at 32°C was statistically significant. Moreover, the long-term effects of DOX became obvious. The increased cytotoxicity of DOX after post-incubation for 48 h was observed in both cell populations: in dividing and in quiescent cells. The difference in the IC_{50} between short-term and long-term assay was statistically significant.

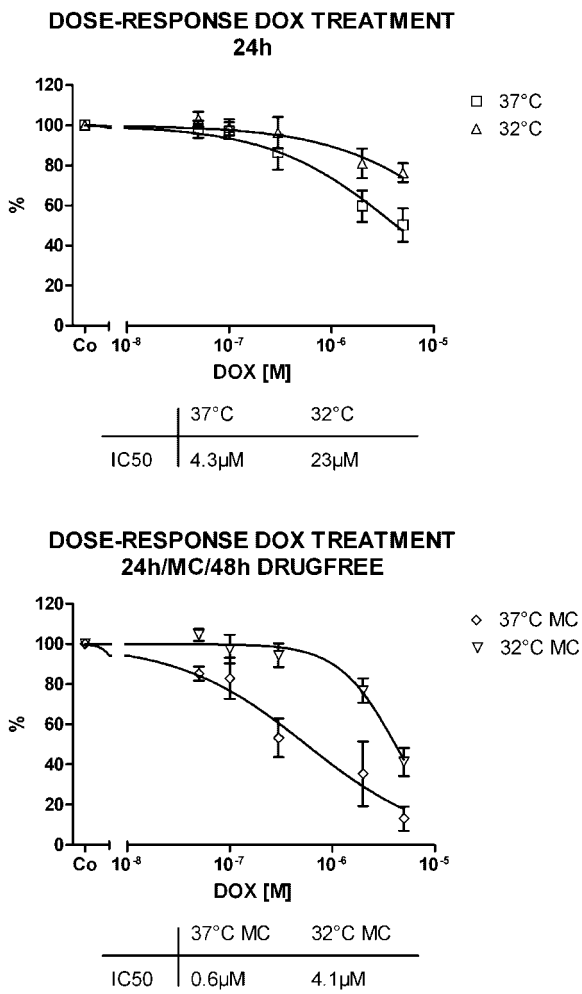

Fig. 8. Cycling cells are more sensitive to DOX treatment than G_1 arrested cells. Cells plated into two microtiter plates were treated with DOX at indicated concentrations for 24 h. Then in the first microtiter plate the number of viable cells was determined by the CellTiterGlo Assay. From the second microtiter plate medium was discarded and fresh medium was added. After post-incubation for 48 h the number of viable cells was determined; MC = medium change. The IC_{50} for DOX was determined. The IC_{50} strongly differed between cells grown at 32 and 37°C after 24 h and after 24 h/MC/48 h drug free. The differences were statistically significant.

Inhibition of Farnesylation of c-Ha-Ras Protein Enhances the Efficacy of Chemotherapy

To assess the potential advantage of the inhibitors of FPTase in the chemotherapeutical treatment of human malignancies, the impact of a combined treatment with FTIs and DOX on transformed rat cells was evaluated. As shown in Figure 9, the inhibition of FPTase potentiated the anti-proliferative effect of DOX. Interestingly, the combined treatment for 24 h increased only slightly the sensitivity of transformed cells to 2 µM DOX. However, the additional inhibition of FPTase during the 48 h post-incubation time significantly enhanced the action of DOX.

Combined Treatment Affects Primarily Cycling but not Quiescent Cells

After showing that combined treatment of transformed rat cells with DOX and FTIs and subsequent treatment with FTIs during the post-incubation period showed very promising growth inhibition, it was fundamental to evaluate the effect of this kind of treatment on non-transformed, quiescent cells. The combined

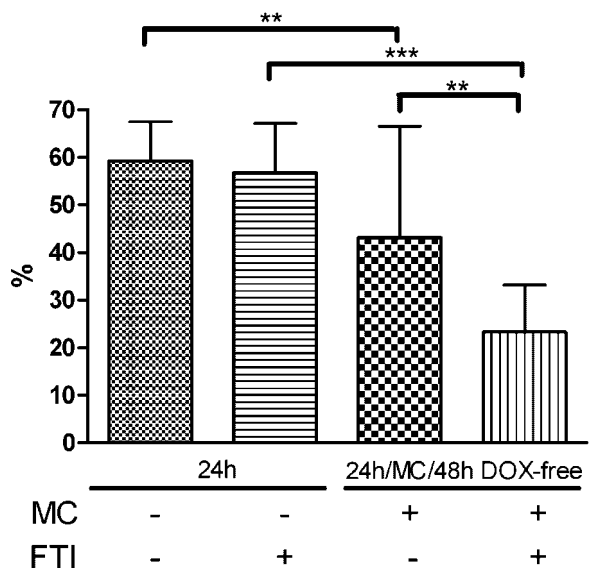

Fig. 9. Combined treatment improves the efficacy of the DOX action. Cells were plated into two microtiter plates and were then treated for 24 h with 2 µM DOX alone, or in combination with 50 µM FTI. Then in the first microtiter plate the number of viable cells was immediately determined by CellTiterGlo Assay. From the second microtiter plate medium was discarded and fresh medium with or without FTI (at a final concentration of 50 µM) was added. After post-incubation for 48 h the number of viable cells was determined by CellTiterLumiGlo Assay; MC = medium change.

treatment with FTIs and DOX had only very little effect on non-cycling cells (data not shown). Obviously, the decrease of the signal transduction efficiency via incubation with FTIs renders the transformed rat cells prone to the action of DOX. Therefore, a strong anti-proliferative effect can be accomplished with lower dose of DOX that clearly should decrease negative effects exerted by DOX on normal, non-malignant cells. This opens a broader therapeutic window that should facilitate the use of DOX and diminish the need to deal with the adverse effects of the drug towards non-neoplastic cells.

DISCUSSION

The Ras proto-oncogene is mutated in a wide variety of cancer cells and typically, a "gain of function" mutation in the *ras* gene is the reason for the tumor-promoting activity of the protein. The mutation leads to a situation where Ras becomes independent of extracellular stimulation and induces sustained growth-promoting signaling. In tumors bearing this kind of activating mutation, a wide range of anti-tumor drugs are not capable of inducing cell-cycle arrest or apoptosis. Therefore, one major rationale 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 prevents the activation of the protein [Gibbs et al., 1994; Prendergast, 2000]. In the present work we used rat cells harboring a *ras* gene with a constitutively activating mutation that, additionally, bear a temperature-sensitive p53 mutant that exhibits a wt phenotype at 32°C but a mutated phenotype at 37°C [Wesierska-Gadek et al., 1996]. Overexpressed p53 protein immediately leads to a cell-cycle arrest when it adopts wt conformation at the restrictive temperature, particularly, when strong signaling from the Ras protein occurs [Peeper et al., 2001]. The described in vitro model enabled us to turn the progression of the cell cycle on or off at will in exactly the same cells just by growing them at the corresponding temperature [Wesierska-Gadek and Schmid, 2000]. This model could potentially help to elucidate basic mechanisms that might lead to strategies to employ drugs that are capable of counter-acting the escape mechanism of mutated Ras together with chemicals that attack the malignant cells at another point while showing only minimal side effects.

Our initial experiments showed that the effect of p53 present in wt or in mutant conformation influenced the cell-cycle progression exactly as predicted, that is, cell-cycle arrest versus unhindered proliferation, respectively. We could also illustrate that mutated Ras protein was biologically active and able to induce progression through the cell cycle. As our results clearly show, the FTI was able to inhibit membrane anchoring of Ha-Ras, whereas the inhibitors of carboxy-methylation were not able to prevent the attachment of Ras to the plasma membrane. As indicated by the pull-down assay in which only activated Ras can be bound by Raf-1, the FTI also rendered the Ras protein biologically inactive. Most importantly, the FTI in combination with the routinely used anti-proliferative chemotherapeutic drug DOX showed a synergistic effect on treated cells. Interestingly, the synergistic effect of FTI became evident after 24 h combined treatment with DOX followed by 48 h post-incubation with FTI alone. During the post-incubation period the addition of FTI reduced the number of viable cells by 20%. This observation is highly important, because it indicates that the described drug administration scheme could be of great interest for the clinical application. Moreover, our results implicate that a longer post-incubation period combined with an inhibition of FPTase could strongly enhance the anti-proliferative action of DOX.

Obviously, the combination of FTI with DOX, a drug that intercalates into DNA and additionally inhibits topoisomerase II was a very promising choice. We could prove that the combination of FTI and DOX had a very strong impact on cycling cells in which p53 has mutant conformation, whereas it has only a negligible effect on non-cycling cells in which p53 adopts wt conformation due to cultivation of cells at 32°C. Interestingly, the kinetics of farnesylation and anchoring of Ha-Ras seems to be rather slow. Within 24 h after start of treatment, only a small part of Ras was unprenylated and cytoplasmic and after an additional 48 h exposure of cells to the drug the ratio of unprenylated Ras strongly increased. This suggests a sustained treatment with the drug to achieve the best results.

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. Most importantly, for the clinical situation the synergistic action should be highly interesting because it opens a wider therapeutic window and the same effect can be accomplished with a significantly lower amount of DOX provided that it is combined with the FTI. Consequently, a decrease in the DOX concentration and the resulting lower toxicity should lead to a significant advantage for the treated patients.

Taken together, the presented data show that our in vitro model based on rat cells bearing mutated Ras as well as a temperature-sensitive p53 protein is well suited to investigate the impact of DOX and a specific FTI on cycling and non-cycling cells under the exactly same genetic background. The specificity of the FTI is also reflected by the finding that other chemicals that inhibit different steps in the post-translational modification of Ras (i.e., carboxy-methylation) do not induce the same anti-proliferative changes when used for treatment of the cells. Our findings suggest that patients bearing a tumor with constitutively activated Ras, should strongly benefit from the combination of certain chemotherapeutic drugs that show a synergistic effect, when used together with FTIs.

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