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# A Combined Treatment of HeLa Cells With the Farnesyl Protein Transferase Inhibitor L-744,832 and Cisplatin Significantly Increases the Therapeutic Effect as Compared to Cisplatin Monotherapy

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**Abstract** Activating mutations of Ras that frequently occur during malignant transformation, enhance growth-promoting signal transduction, allowing cells to bypass stringent control of cell cycle progression, thereby rendering them highly proliferative. Abundantly expressed c-Ha-ras protein in human cervical HeLa cells is farnesylated and attached to the plasma membrane, inducing enhanced signal transduction. Exposure of HeLa cells to cisplatin very efficiently inhibits cell proliferation and induces apoptosis. Unfortunately, high doses of cisplatin are strongly cytotoxic, therefore, an alternative therapeutic strategy allowing dose reduction of cisplatin by inhibition of farnesylation could increase the curative effects of cisplatin, thereby benefiting cancer patients. We used two inhibitors of farnesyl protein transferase (FPTase), FTI, and L-744,832, to sensitize HeLa cells to the action of cisplatin. The combined administration of cisplatin and inhibitors of FPTase increased the cytostatic potency of cisplatin. L-744,832 exhibited a stronger synergistic effect in combination with cisplatin than FTI. Moreover, the efficiency of the combined therapy strongly depended on the treatment regimen: The highest efficiency was achieved after combined treatment for 24 h and post-incubation with an inhibitor of FPTase for 48 h. Following this optimized treatment, apoptosis was induced in approximately 50% of HeLa cells treated with 1 μM cisplatin, representing approximately a threefold increase as compared to cisplatin monotherapy. Combined treatment of HeLa cells with cisplatin and inhibitors of FPTase significantly increases the efficacy of the therapy and allows to reduce the dose of cisplatin. Importantly, best therapeutic effects can be achieved by post-treatment with inhibitors of FPTase. J. Cell. Biochem. 104: 189–201, 2008. 

**Key words:** apoptosis; c-Ha-ras; cisplatin; HeLa cells; farnesyl protein transferase

Cisplatin is a widely used drug for the treatment of various types of human malignancies. However, cisplatin is strongly cytotoxic at higher concentrations and therefore, a treatment regimen employing lower doses would minimize side effects, thereby benefiting chemotherapy

Abbreviations used: Akt/PI3-kinase, (phosphatidyl-inositol 3-kinase); Casp, caspase; Co, control; Conc, concentration; CP, cisplatin; DAPI, 4,6-diamidino-2-phenylindole; FPTase, farnesyl protein transferase; FTI, farnesyl protein transferase inhibitor; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GTPase, guanosine triphosphatase; h, hour; IAPs, inhibitor of apoptosis proteins; MB; membrane-bound; MC; medium change; 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 protein; RBD, Raf binding domain; ts, temperature-sensitive; SD, standard deviation; vs., versus; WCL, whole cell lysate; WT, wild-type.

The authors declare that they have no competing interests.

JWG designed and established the project, coordinated and supervised the work. JWG also was responsible for acquisition of material and drafted the initial version of the manuscript. MPK performed the in vitro experiments, prepared most of the figures, and made the calculations for statistical analysis. GS was involved in the design of the project and wrote and formatted most of the manuscript, including the final version. All authors were involved in data interpretation and critically read and approved the final manuscript.

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patients. Cisplatin inhibits proliferation of human HeLa cervix carcinoma cells in a dose- and time-dependent manner [Wesierska-Gadek et al., 2002; Schloffer et al., 2003] through cell cycle arrest in $G_1$ phase and via the induction of apoptosis by membrane death receptor-mediated and mitochondria-mediated pathways [Wesierska-Gadek et al., 2002; Yim et al., 2006]. The anti-proliferative and pro-apoptotic actions of cisplatin become evident only at relatively high concentrations. However, cisplatin-treated tumor cells frequently develop a resistance against the drug during chemotherapy [Liang et al., 2006], predominantly, through activation of growth-promoting and anti-apoptotic pathways.

One well-documented tumorigenic mechanism that occurs early and very frequently during malignant transformation is caused by activating "gain of function" mutations of Ras, a cellular protein with intrinsic guanosine-triphosphatase (GTPase) activity whose enzymatic functions are usually turned on via extracellular growth factor stimulation [Janda et al., 2002]. Mutated Ras enhances growth-promoting signal transduction and induces sustained activation of the Akt/PI3-kinase (phosphatidyl-inositol 3-kinase) pathway even in the absence of extracellular growth factor-mediated stimulation, thereby allowing cells to bypass cell cycle control and to acquire an increased proliferative capacity and ultimately resulting in enhanced cell motility and increased invasiveness of tumors [Bishop et al., 2003]. Elevated expression of c-Ha-Ras was also found in human cervical HeLa cancer cells. The highly expressed c-Ha-Ras becomes post-translationally modified by farnesylation and subsequently attaches to the plasma membrane, thereby enhancing signal transduction via Akt. Most importantly, inhibition of farnesylation completely prevents the attachment of c-Ha-Ras to the proximal side of the plasma membrane, thereby reversing the effect of the activating mutation [Lerner et al., 1995]. Several inhibitors of FPTase (FTI) are already in clinical trials where the high expectations could only partly be fulfilled [Caponigro et al., 2003; Doll et al., 2004; Appels et al., 2005; Margaritora et al., 2005]. Considering the increased expression of c-Ha-Ras in HeLa cells, we addressed the question, whether inhibition of its farnesylation would sensitize HeLa cells to cisplatin and augment its curative potential.

In vivo, cancer cells are exposed to a high concentration of cisplatin within the first 24 h after administration of the drug and then, due to metabolic clearance, the concentration of cisplatin strongly decreases. In order to mimic this in vivo situation in a cell culture model, we exposed HeLa cells to cisplatin for 24 h and subsequently incubated the cells in drug-free medium for 48 h. Tumor cells were either treated solely with cisplatin, or, to increase the anti-proliferative potential, they were incubated with a combination of cisplatin and one of two inhibitors (FTI or L-744,832) of farnesyl protein transferase (FPTase), an enzyme catalyzing the covalent attachment of farnesyl-PP residues to target proteins. The combined administration of cisplatin and inhibitors of FPTase clearly increased the cytostatic effect of cisplatin. The inhibitor L-744,832 exhibited a stronger synergistic effect in combination with cisplatin than FTI. Moreover, the efficacy of the combined therapy strongly depended on the time course of administration of the drugs. The highest efficiency was achieved after combined treatment for 24 h and post-incubation with the inhibitor for 48 h. Following this treatment schedule, apoptosis was induced in approximately 50% of HeLa cells when treated with 1 μM cisplatin. This represents an approximately threefold increase in cell death as compared to cisplatin-mediated effects alone.

Thus, our results show that the combined treatment of HeLa cells with cisplatin and inhibitors of FPTase increase the therapeutic effect and allow to reduce the dose of cisplatin. Most importantly, optimal therapeutic effects can be achieved by post-treatment of the tumor cells with inhibitors of FPTase. The significant reduction of the cisplatin concentration, when combined with an inhibitor of FPTase, should strongly diminish the adverse side effects of the drug in chemotherapy, thereby providing an important benefit to the big group of patients that receive cisplatin as part of their chemotherapeutic intervention.

## MATERIALS AND METHODS

### Cells

The human cervical carcinoma cell line $HeLaS_3$, obtained from American Type Culture Collection (ATCC), was cultured in RPMI medium supplemented with 10% fetal calf serum. Cells were grown up to 60–70%

confluence and then treated with allocated drugs. Rat embryo fibroblasts transformed with *ts* $p53^{135val}$ + *c-Ha-ras* were used as a positive control [Wesierska-Gadek et al., 1996a]. The establishment of transformed rat cell clones was described previously in detail [Wesierska-Gadek et al., 1996b, 2006].

### Drugs

We used cisplatin (Lachema, Czech Republic) and two different inhibitors of FPTase: the farnesyltransferase inhibitor [*N*-[2(S)-[2-(R)-amino-3-mercapthopropylamino]-3-methylbutyl]-Phe-Met-OH] (FTI) from Alexis Biochemicals (Lausen, Switzerland) and (2S)-2-[[(2S)-2-[(2S,3S)-2-[(2R)-2-amino-3-mercaptopropyl] amino]-3-methylpentyl]oxy]-1-oxo-3-phenylpropyl]amino]-4-(methylsulfonyl)-butanoic acid 1-methylethyl ester (L-744,832) from Calbiochem (Calbiochem-Novabiochem Corp., La Jolla, CA). A stock of FTI was diluted in an aqueous solution containing 1% DMSO. The stock solution of L-774,832 was prepared in DMSO. Aliquots of stock solutions were protected from light and stored until use at −20°C.

### Cell Treatment

Exponentially growing cells were exposed to cisplatin at a final concentration ranging from 0.1 to 40 μM for indicated periods of time. In some experiments cells were additionally treated with one of two inhibitors of farnesyltransferase: FTI or L-744,832 at a final concentration of 25 μM.

### Antibodies

Monoclonal anti-PCNA antibodies (clone PC-10) were purchased from Oncogene Research Products (Cambridge, MA) and anti-Ran antibodies were from Transduction Laboratories (Lexington, KY). Monoclonal anti-p21-c-Ha-Ras antibodies were from DAKO A/S (Glostrup, Denmark) and anti-Actin antibodies were from ICN (ICN Biomedicals, Aurora, OH). Compatible secondary antibodies were obtained from Amersham International (Little Chalfont, Buckinghamshire, England).

### Determination of the Number of Viable Cells

The sensitivity of exponentially growing $HeLaS_3$ cells to increasing concentrations of cisplatin alone, or in combination with FPTase inhibitors, was determined with the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). The CellTiter-Glo Luminescent Cell Viability Assay, generating luminescent signals, is based on quantification of the cellular ATP levels [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2004, 2005b]. Cells ($5 \times 10^3$ cells/well) were plated into 96 well microtiter plates (two plates for each condition). One day after plating, cells were exposed to drugs 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 post-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 provided 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, or in the presence of an inhibitor of FPTase, allowed the evaluation of the long-term effects of the treatment. 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 ± SD (bars) of replicates from at least four experiments. Statistical significance of the results was evaluated using Bonferroni's Multiple Comparison Test.

### Determination of Caspase-3/7 Activity

The activity of both caspases was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega Corporation) which uses the caspase-3/7 substrate rhodamine 110, bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R100) [Wesierska-Gadek et al., 2005b]. This compound exists as a profluorescent substrate prior to its turnover. Upon cleavage and removal of the DEVD peptide by caspase-3/7 activity and excitation at 499 nm, the removed rhodamine 110 group becomes intensely fluorescent. $HeLaS_3$ cells were plated in 96-well microtiter plates. One day after plating, cells were exposed for 24 h to increasing cisplatin concentrations alone, or in combination with FTPase inhibitors. Thereafter, culture supernatant was transferred into another microtiter plate to separately determine the caspase activity in cells and in culture medium. Then an equal volume of caspase substrate was added and samples were incubated at 37°C for different periods of time to assess the best signal-to-background ratio [Wesierska-Gadek, 2005; Wesierska-Gadek et al., 2005b]. The

fluorescence was measured at 485 nm. Culture medium (no-cell background) was used as a blank. Fluorescence was measured in the Wallac 1420 Victor. Each point represents the mean ± SD (bars) of four values from one representative experiment.

### 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] from Vindelov et al. [1983]. The cells were detached from substratum by limited trypsinization, and then all cells were harvested by centrifugation and washed in PBS. Aliquots of $1 \times 10^6$ 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 1 h incubation of the cells at +4°C in the dark.

### Detection of Apoptotic Cells by CytoDEATH Staining

Apoptotic cells were detected by M30 CytoDEATH monoclonal antibodies (Roche Molecular Biochemicals) recognizing caspase-3 cleaved cytokeratine 18. Apoptotic cells were detected in situ by indirect immunofluorescence microscopy and were additionally quantified by flow cytometric analysis [Wesierska-Gadek et al., 2005b]. For microscopic analysis cells were plated on slides in plastic chambers and appropriately cultivated. After treatment for indicated time, cells were washed three times in PBS, immediately fixed in ice-cold methanol, and stained. The fixed cells were incubated according to the manufacturer's protocol with the fluorescein-coupled monoclonal antibodies M30. For visualization of nuclei, cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) dissolved in PBS at a final concentration of 1 μg/ml [Wesierska-Gadek et al., 1999; Wesierska-Gadek and Schmid, 2001]. Cells were inspected under a fluorescence microscope (inverted microscope Eclipse TE300, Nikon Corporation, Tokyo).

### Cell Fractionation

Cells were harvested, washed with PBS, and resuspended in ice-cold hypotonic buffer supplemented with protease inhibitors [Wesierska-Gadek et al., 2005a]. Cells were swollen for 10 min. Then the cell suspension was vigorously vortexed and centrifuged at 5,000*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) membranes. 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; $M_w = 26$ kDa) is a small GTPase of the Ras superfamily.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism and significance levels were evaluated using Bonferroni's Multiple Comparison Test.

## RESULTS

### Exposure of HeLa Cells to Cisplatin Inhibits Cell Cycle Progression and Induces Apoptosis

In agreement with previously published data, treatment of HeLa cells for 24 h with increasing cisplatin concentrations resulted in a clear reduction of the number of viable cells in a time- and concentration-dependent manner (Fig. 1). Determination of the DNA concentration in single cells showed that cisplatin arrests cells in $G_1$ phase (Fig. 2A) and at higher concentrations it induces apoptosis (Fig. 2B). The apoptosis rate and its kinetics were determined by different methods: flow cytometric determination of the number of the sub-$G_1$ cells and of the number of cells stained by CytoDEATH (Fig. 2B) as well as measurement of caspase-3/7 activity (Fig. 3).

To mimic the in vivo situation (treatment of patients), we exposed HeLa cells to different cisplatin concentrations for 24 h and after medium change, cells were post-incubated in a drug-free medium for a further 48 h. The comparison of the action of cisplatin directly after 24 h treatment and 48 h post-incubation

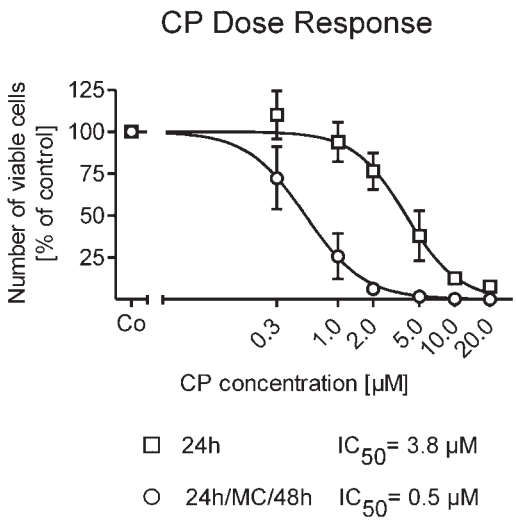


**Fig. 1.** Reduction of the number of viable HeLa cells after treatment with cisplatin. Exponentially growing HeLa cells plated in 96-well microtiter plates were treated for 24 h with increasing concentrations of cisplatin (CP). The number of viable cells was determined directly after treatment (□ 24 h) and additionally, after medium change (MC) and post-incubation for 48 h in a drug-free medium (○ 24 h/MC/48 h). The graph represents mean values from seven independent experiments, each performed at least in quadruplicates. $IC_{50} = 3.8$ μM cisplatin after 24 h treatment; $IC_{50} = 0.5$ μM cisplatin after treatment for 24 h, medium change and post-incubation for 48 h. Error bars represent values of SD. The difference of $IC_{50}$ values is statistically very highly significant ($P < 0.0001$).

revealed a big difference between short- and long-term effects of cisplatin treatment (Fig. 1). The $IC_{50}$ value measured directly after 24 h treatment was 3.8 μM cisplatin and decreased nearly eightfold after post-incubation for 48 h (0.5 μM cisplatin). These results implicate that cells appearing to be viable after 24 h treatment with lower cisplatin doses, have been irreversibly damaged and become apoptotic during the post-incubation period. Cells were unable to proliferate and died during further cultivation.

### The Effect of Cisplatin on c-Ha-Ras in HeLa Cells

Comparison of c-Ha-Ras expression of frequently used human cell lines revealed a strong c-Ha-Ras protein band in human HeLa and HL-60 cancer cells (Fig. 4A). Transformed rat cells over-expressing mutated human c-Ha-Ras were loaded as a positive control. As shown in Figure 4A, c-Ha-Ras protein was detected in four out of seven tested human cancer cell lines. The anti-c-Ha-Ras antibody did not show any signal in the sample of human A549 cells that are known to over-express Ki-Ras (data not shown). This indicates that the monoclonal antibody used for immunoblotting was very

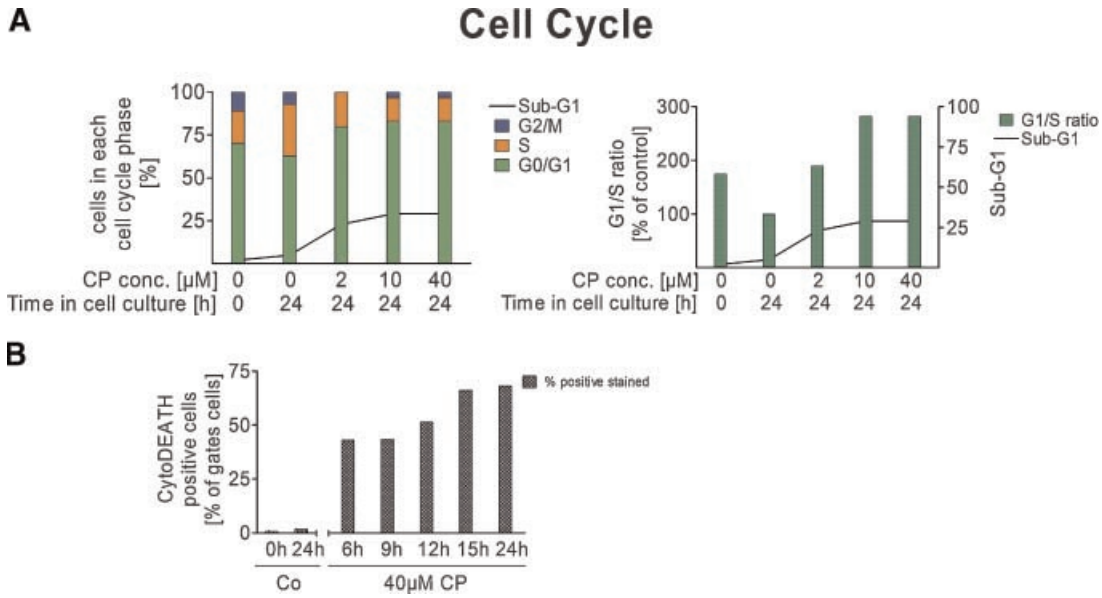


**Fig. 2.** Cisplatin at higher concentrations blocks HeLa cells in $G_1$ phase of the cell cycle and induces apoptosis. HeLa cells were treated with 40 μM cisplatin (CP) for the indicated period of time. Control and cisplatin-treated cells were detached from substratum by limited trypsin digestion and single cell suspension was used for propidium iodide (A) and CytoDEATH (B) staining. **A**: DNA content in single cells was measured using the Becton Dickinson FACScan flow cytometer. The distribution of HeLa cells in distinct cell cycle phases and the $G_1$/S ratio are shown. The values represent a mean of two replicates. **B**: PBS-washed cells were fixed according to the manufacturer's recommendations and stained using the fluorescein-coupled CytoDEATH antibody selectively recognizing caspase-3 cleaved cytokeratine 18. Positively stained cells were quantified by flow cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

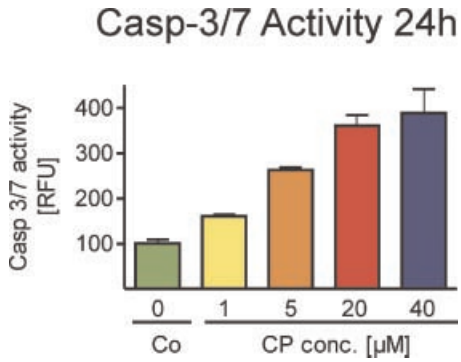


**Fig. 3.** Activation of caspase-3 in apoptotic HeLa cells after longer exposure to cisplatin. Exponentially growing HeLa cells plated in a multiwell plate were treated for 24 h with cisplatin (CP) at indicated concentrations. Activity of caspase-3/7 was determined in triplicates in a multiwell plate using the APO-One Assay. Medium was used as a blank. The caspase-3/7 activity [relative fluorescence units (RFU)] ± SD was normalized to the values of control cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

specific and did not cross react with other members of the Ras protein family. We have previously observed that cisplatin negatively affected the expression of some cellular and virally encoded proteins in HeLa cells [Wesierska-Gadek et al., 2002]. Therefore, we examined the effect of cisplatin on the level of c-Ha-Ras protein. As shown in Figure 4B, exposure of HeLa cells to 20 μM cisplatin for 15 h and for 24 h did not affect the total cellular amount of c-Ha-Ras protein. However, the combined treatment with FTI resulted in a shift of the reactive c-Ha-Ras protein band (Fig. 4B). The change of the electrophoretic mobility of the c-Ha-Ras protein is a consequence of the inhibition of FPTase activity. Unmodified c-Ha-Ras protein is neither cleaved by specific protease nor carboxymethylated or palmitoylated and therefore, its size and hydrophobic features differ from those of the processed form. After inhibition of FPTase activity for 24 h the c-Ha-Ras protein band was shifted. The electrophoretic shift is attributable to the diminution of c-Ha-Ras processing [Wesierska-Gadek et al., 2006, 2007]. This effect became more pronounced after exposure of HeLa cells to the higher concentration of FTI for 24 h (Fig. 4B, last lane on the right).

### Prevention of Plasma Membrane Attachment of De Novo Synthesized c-Ha-Ras Protein After Treatment of HeLa Cells With Inhibitors of FPTase

To attain the full biological activity, de novo synthesized c-Ha-Ras protein has to be processed and finally to be attached to the plasma membrane. We monitored the effect of two distinct FPTase inhibitors on the subcellular distribution of c-Ha-Ras protein in HeLa cells. In untreated control cells, the protein was localized in the plasma membrane fraction. The inhibition of FPTase activity by FTI and/or L-744,832 resulted in the loss of its attachment to the plasma membrane. After exposure of HeLa cells to the inhibitors for 24 h, a substantial fraction of c-Ha-Ras became soluble and was detected in the cytosol (Fig. 5). Thus, the results evidence that endogenous c-Ha-Ras protein in HeLa cells is properly processed and attached to the plasma membrane.

### Negligible Short-Term Effect of the Combined Treatment With Cisplatin and FTIs on the Viability of HeLa Cells

Considering the fact that inhibition of FPTase resulted in the reduction of the proper localization of c-Ha-Ras protein, we determined the effect of FPTase inhibitors alone, or in combination with cisplatin, on the number of viable cells after treatment for 24 h. Exposure of HeLa cells to low cisplatin concentrations (0.3 and 1 μM) for 24 h did not reduce the number of viable cells (Fig. 1). 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 10% (Fig. 6). The combined treatment with 0.3 μM cisplatin and L-744,832 for 24 h only slightly affected the viability of the cells. Simultaneous administration of L-744,832 and 1 μM cisplatin reduced the number of living cells by 20% as compared with cisplatin alone. Moreover, it became obvious that L-744,832 was more efficient than FTI. The addition of inhibitors of FPTase 6 h prior to the onset of cisplatin treatment had no therapeutic effect. These results show that simultaneous treatment of cells with cisplatin and FPTase inhibitors for 24 h does not substantially increase the effect of cisplatin (Fig. 6).

### FTI Inhibitors Reduce the Cisplatin-Induced Accumulation of S-Phase-Arrested Cells

Determination of the DNA content in single cells by flow cytometry revealed detailed information about changes of the cell cycle status of HeLa cells upon drug treatment. Exposure of HeLa cells to L-744,832 for 24 h did not affect their distribution in cell cycle phases as compared to

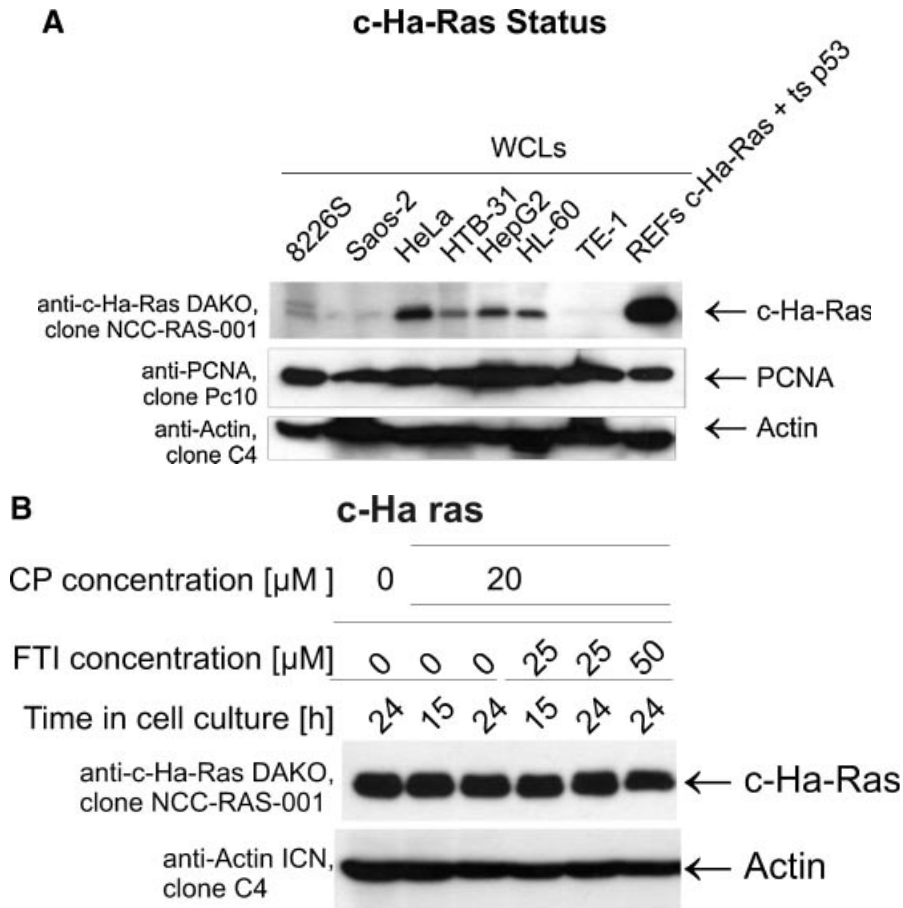


**Fig. 4.** Exposure of HeLa cells to cisplatin did not affect the cellular levels of c-Ha-Ras protein. **A**: Untreated, exponentially growing cancer cell lines were harvested and lyzed. Whole cell lysates (WCLs) were loaded on 15% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein 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. **B**: Exponentially growing HeLa cells were treated for 24 h with 20 μM cisplatin (CP) alone or in combination with FTI and then lyzed. WCLs were loaded on 15% SDS gels. Conditions of separation and immunoblotting were described in detail in (A). The equal protein loading was checked by immunoblotting with anti-actin antibodies.

the 24 h control cells. Although the exposure of HeLa cells to low cisplatin doses for 24 h was not directly cytotoxic and did not kill the cells, it affected their cell cycle status. The population of cells in S-phase markedly increased. The treatment with 1 μM cisplatin increased the number of S-phase cells by 100% (Fig. 7). This result is surprising because the treatment with cisplatin at higher concentrations inhibits the cell cycle progression in $G_1$ phase [Wesierska-Gadek et al., 2002; Schloffer et al., 2003].

### Synergistic Effect of the Inhibition of FPTase During the Post-Incubation Period on the Action of Cisplatin

The exposure of cisplatin-treated HeLa cells to inhibitors of FPTase during the post-

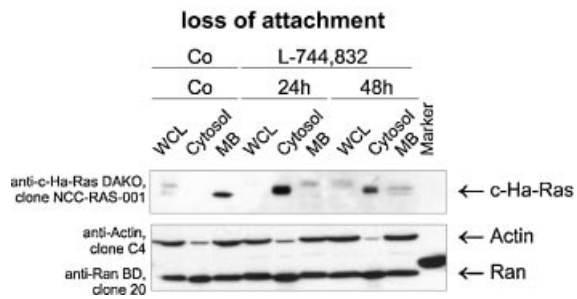


**Fig. 5.** Loss of the attachment of de novo synthesized c-Ha-Ras protein to plasma membrane after treatment of HeLa cells with inhibitors of FPTase. HeLa cells treated with the FPTase inhibitor L-744,832 for 24 h and for 48 h were collected and fractionated to separate soluble (cytosol) and membrane-bound (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 4A.

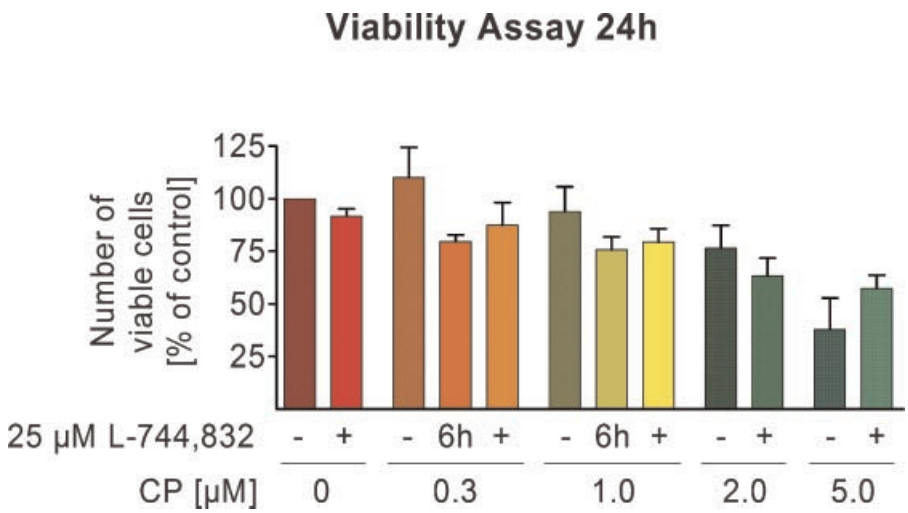


**Fig. 6.** Weak effect of a simultaneous treatment of HeLa cells with cisplatin and inhibitors of FPTase on the number of living cells. HeLa cells were treated with low cisplatin (CP) doses for 24 h alone, or in combination with L-744,832 (+). In some samples L-744,832 was added 6 h prior to the onset of cisplatin treatment (6 h). After treatment, the number of living cells was determined using the CellTiter-Glo Assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

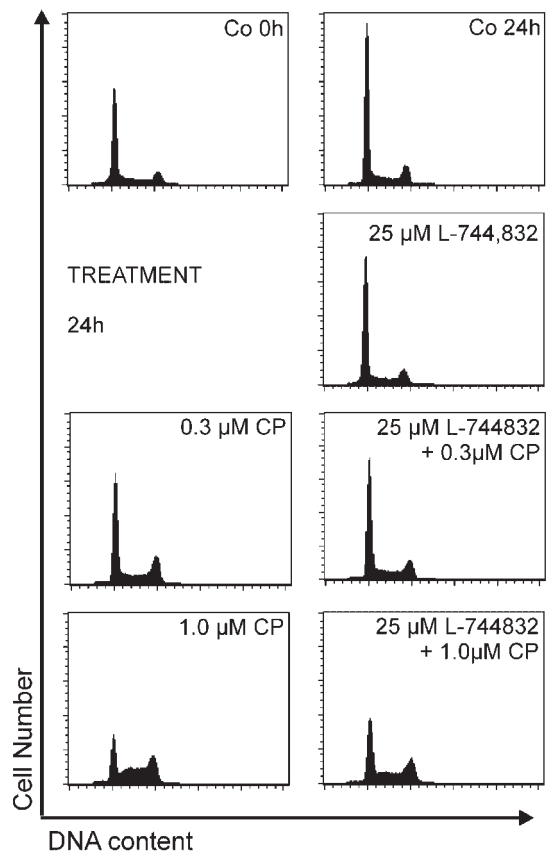


**Fig. 7.** Combined treatment of HeLa cells with cisplatin and an inhibitor of FPTase for 24 h prevents accumulation of S-phase cells. HeLa cells treated with drugs for 24 h as indicated, were collected and stained with propidium iodide. Conditions of staining and measurement as described in Figure 2.

incubation for 48 h strongly diminished the number of viable cells HeLa cells (Fig. 8). The $IC_{50}$ value measured after 24 h treatment with cisplatin alone and post-incubation for 48 h in a drug-free medium was 0.5 μM cisplatin and decreased nearly sixfold after combined treatment and post-incubation for 48 h in the presence of L-744,832 (0.08 μM cisplatin; Fig. 8B). The reduction of the $IC_{50}$ values was statistically very highly significant ($P < 0.0001$). The flow cytometric determination of DNA content in single cells delivered the explanation of the increased sensitivity of HeLa cells to the combined treatment.

### Effect of the Combined Treatment With Cisplatin and FPTase Inhibitors on the Cell Cycle Status of HeLa Cells

The flow cytometric monitoring of the DNA content in single cells revealed that inhibition of FPTase during the post-incubation period affected the cell cycle status of cisplatin-treated HeLa cells and strongly promoted the induction of apoptosis. The substantial decrease of the population of S phase-arrested cells coincided with the accumulation of $G_2$-arrested cells. This is reflected by the increase of the $G_2/G_1$ ratio (Fig. 9B). The population of sub-$G_1$ cells representing cells undergoing apoptosis increased approximately threefold in the presence of FPTase inhibitors (Fig. 9B).

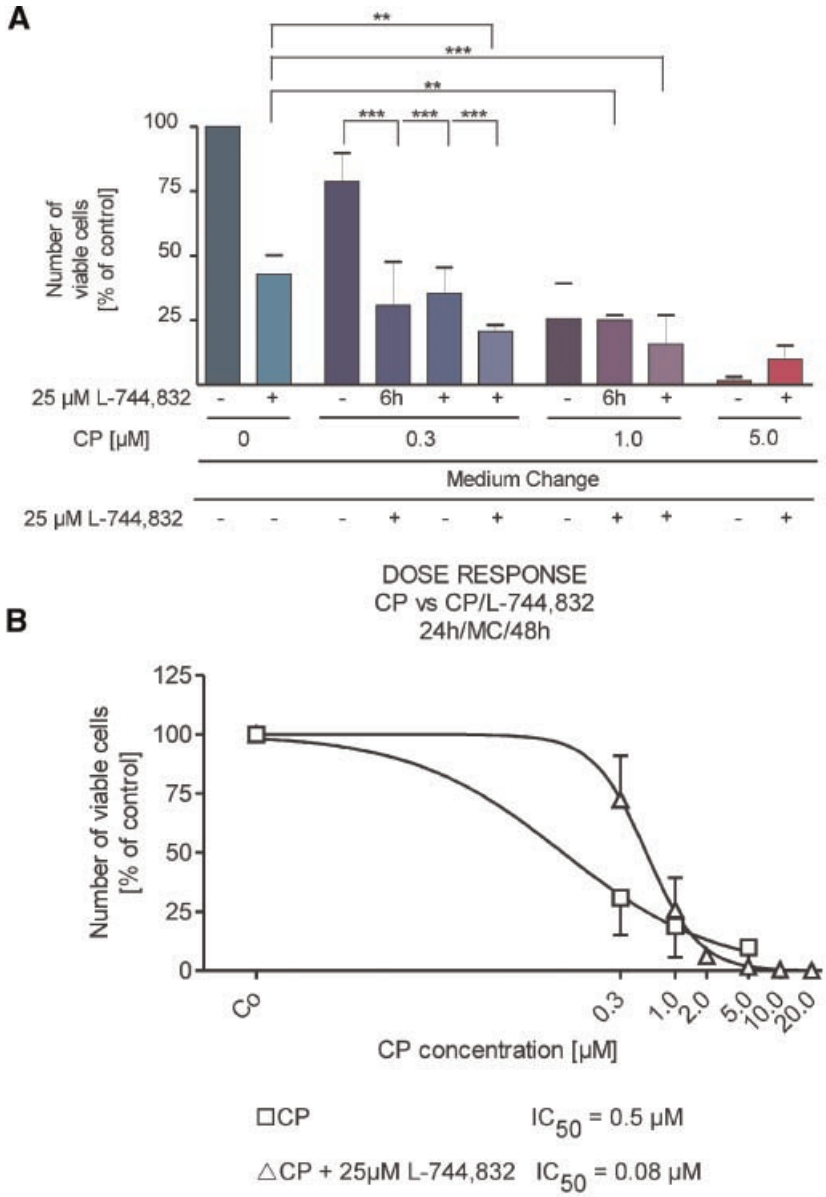


**Fig. 8.** Inhibition of FPTase during post-incubation after cisplatin treatment of HeLa cells strongly enhances the action of cisplatin. **A**: HeLa cells were treated with low cisplatin (CP) doses for 24 h alone, or in combination with L-744,832 (+). In some samples L-744,832 was added 6 h prior to the onset of cisplatin treatment (6 h). After treatment medium was changed (MC) and cells were post-incubated in drug-free medium or in the presence of L-744,832 for 48 h. The number of living cells was determined using the CellTiter-Glo Assay. **B**: HeLa cells were treated with low cisplatin (CP) doses for 24 h alone, or in combination with L-744,832 (+). After treatment medium was changed (MC) and cells were post-incubated in drug-free medium or in the presence of L-744,832 for 48 h. The number of living cells was determined using the CellTiter-Glo Assay. $IC_{50} = 0.5$ µM cisplatin after treatment with cisplatin alone; $IC_{50} = 0.08$ µM cisplatin after combined treatment with L-744,832. Error bars represent values of SD. The difference of $IC_{50}$ values is statistically very highly significant ($P < 0.0001$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Inhibitors of FPTase Combined With Cisplatin Induce Apoptosis in HeLa Cells

The appearance of approximately 50% sub-$G_1$ cells during the inhibition of FPTase in cisplatin-treated cells was a strong indication for apoptosis. As shown in Figure 10, the inhibitors of FPTase strongly induced the activity of caspase-3/7. This was additionally confirmed by the detection of activated caspases-3 and 9 as well as by caspase-3-mediated cleavage of PARP-1 by immunoblotting and by CytoDEATH staining (data not shown).

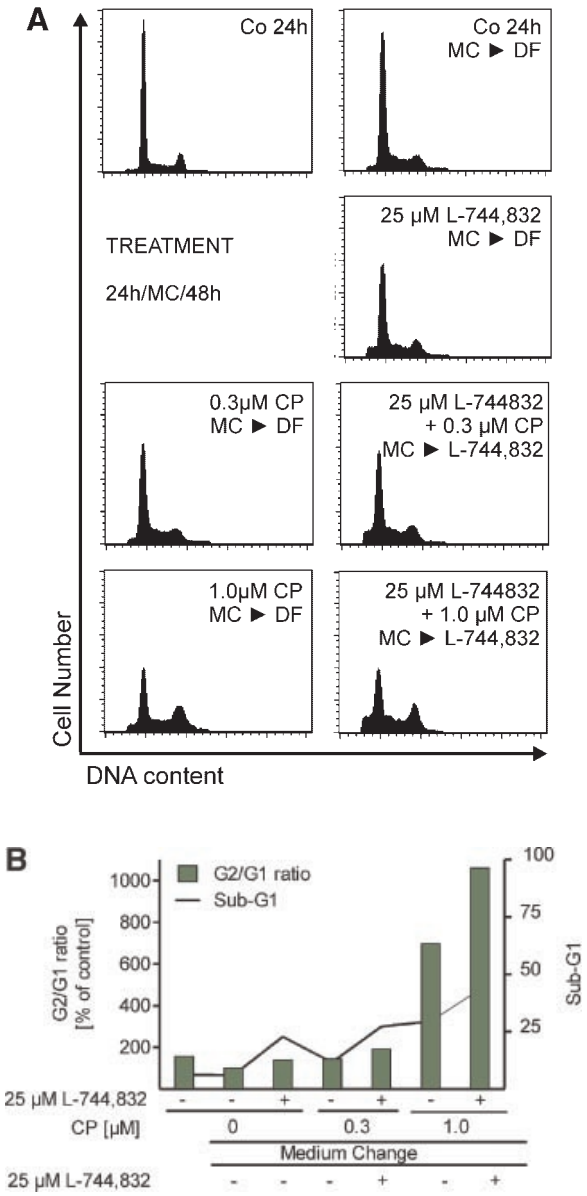


**Fig. 9.** Long-term effects of the combined treatment of HeLa cells with cisplatin and an inhibitor of FPTase on the cell cycle status. HeLa cells were treated with low cisplatin doses for 24 h alone, or in combination with L-744,832. After treatment, medium was changed and cells were post-incubated in drug-free medium or in the presence of L-744,832 for 48 h. Thereafter, cells were harvested and stained with propidium iodide. Conditions of staining and measurement as described in Figure 2. **A**: DNA profile. **B**: Diagram showing the changes of $G_2/G_1$ ratio and the amount of sub-$G_1$ cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

## DISCUSSION

Cisplatin is one of the most commonly used chemotherapeutic drugs, especially for the treatment of cervical carcinoma. However, the concentrations of cisplatin that are required to induce a sufficient apoptotic response in cancer cells are rather high and therefore, also pose a risk for healthy cells. To be able to reduce the necessary amount of cisplatin, combination therapies, for example, with radiation [Koivusalo et al., 2002] or with additional drugs, have been conducted with some success. In the present work we show an approach to combine the effect of two synergistically acting chemotherapeutic drugs: cisplatin and an inhibitor (FTI or L-744,832) that prevents the farnesylation of the over-expressed c-Ha-Ras protein, thereby reverting its growth-promoting and anti-apoptotic properties. Most importantly, FTIs have demonstrated additive or synergistic effects when used in combination with other drugs [Moasser et al., 1998; 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]. However, under some circumstances, the use of an FTI (BZA-5B) can also result in an increased resistance of cancer cells to cisplatin [Fokstuen et al., 1997]. The exact underlying mechanistical background for this finding still has to be elucidated. Possibly, also the 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].

Like in many other cancer cells, c-Ha-Ras is highly expressed in HeLa cells and disabling the signal transduction through the protein appears to be a very promising target to facilitate the induction of apoptosis in these cells. Our results clearly indicate that this strategy works as expected in vitro and suggests to confirm the findings in an in vivo setting. The comparison of the early and late effects of cisplatin action revealed that cells appearing to be viable after 24 h treatment with lower cisplatin doses, have been irreversibly damaged and become apoptotic during the post-incubation period. Cells were unable to proliferate and died during further cultivation for 48 h in a drug-free medium. These results also indicate that the post-incubation time window offers an excellent opportunity for treatment with non-genotoxic agents that inhibit signal transduction processes and/or cell cycle progression to enhance the initial effect of cisplatin. The

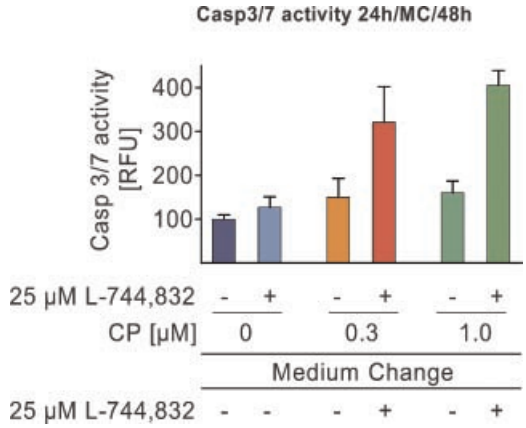


**Fig. 10.** Inhibition of FPTase during post-incubation after cisplatin treatment of HeLa cells strongly activates caspase-3. Cells were treated with cisplatin and L-744,832 for 24 h. Then the medium was changed (MC) and cells were post-incubated in drug-free medium or in the presence of L-744,832. The activity of cellular caspase-3/7 was determined in quadruplicates using the APO-One Assay. Medium was used as a blank. The number of viable cells was determined in the same microtiter plate by CellTiter-Glo. The caspase-3/7 activity [relative fluorescence units (RFU)] ± SD was normalized to the number of viable cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

comparison of the different treatment schedules (e.g., pretreatment with L-744,832 for 6 h or concomitant onset of treatment) implicate that the best results were obtained after treating the cells with a combination of cisplatin and L-744,832 for 24 h, and then changing to a post-incubation of the cells with L-744,832 alone. When applying this schedule, the ratio of apoptotic cells increases nearly threefold as compared to the cisplatin-only treatment. The $IC_{50}$ for cisplatin-only treatment was nearly sixfold higher (0.5 vs. 0.08 μM) than for the combined treatment together with L-744,832. The significantly lower concentration of cisplatin should strongly diminish adverse side-effects originating from high-dose cisplatin treatment in the clinical situation. The synergistic effect of both drugs and the post-treatment with the FTI should also open a much wider therapeutic window that should result in a clear benefit for patients receiving chemotherapy [Wesierska-Gadek et al., 2006, 2007]. The process of apoptosis, initiated in HeLa cells after administration of cisplatin, is executed in two phases [Horky et al., 2001]. During an early apoptotic stage an activation of caspase-9 and caspase-3 accompanied by proteolytic cleavage of poly(ADP-ribose) polymerase-1 and formation of the apoptosome was observed [Horky et al., 2001]. Considering the fact that even activated caspases can be inhibited by cellular inhibitor of apoptosis proteins (IAPs), the inhibition of the pro-survival signal transduction pathway after prevention of proper processing of c-Ha-Ras protein could facilitate the execution of cisplatin-induced apoptosis in HeLa cells.

L-744,832 was shown to prevent the up-regulation 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]. Additionally, L-744,832 sensitizes cancer cells in vivo to radiation therapy by reducing hypoxia in the tumor tissue [Cohen-Jonathan et al., 2001]. This is highly important considering the finding, that hypoxia protects tumor cells from radiation as well as 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. A very recent paper 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 marked difference between the regulation of c-Ha-Ras and wild-type Ras could also explain why the toxicity of FTIs towards wild-type cells is quite low.

Taken together, our data clearly indicate that a combination therapy employing cisplatin and L-744,832 shows a synergistic effect of the two drugs and allows to reduce the adverse side effects of cisplatin. The effect is further enhanced when cells are treated with both drugs, simultaneously, and subsequently incubated with L-744,832, alone. Our promising findings suggest that a treatment regimen that makes use of a combination of the two drugs under an optimized schedule, should be very effective and yet less burdening for cancer patients.

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