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**Chemotherapy Compounds in Cervical Cancer Cells Primed by Reconstitution of p53
Function After Short Interfering RNA-mediated Degradation of Human Papillomavirus
18 E6 mRNA. Opposite Effect of siRNA in Combination With Different Drugs.**

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Nonstandard abbreviations: DMEM, Dulbecco's modified Eagle's medium; HPV, Human Papillomavirus; HeLa CMV, HeLa cell line carrying an empty plasmid; HeLa DD, HeLa cell line expressing a truncated dominant negative p53 protein; MDM2, murine double minute 2 protein; pRb, retinoblastoma protein; qRT-PCR, quantitative real-time PCR; siRNA, short interfering RNA; TRITC, tetramethylrhodamine isothiocyanate; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

Abstract

Constant expression of E6 and E7 mRNA by high-risk human papillomaviruses (HPV) abrogates p53 and pRb function, respectively, and is essential for the development of cervical cancer. Despite E6, some chemotherapy drugs can stabilize p53 in cervical cancer cells. It is not known how chemotherapy-induced p53 activation and cytotoxicity is affected when the amount of E6 mRNA is decreased before the drug treatment. Here, HPV 18 positive HeLa cervical cancer cells were transfected with short interfering RNA (siRNA) molecules targeting HPV 18 E6 mRNA before treatment with carboplatin, cisplatin, doxorubicin, etoposide, gemcitabine, mitomycin, mitoxantrone, oxaliplatin, paclitaxel and topotecan. Transfection with siRNA was followed by nuclear accumulation of p53, but the effect was transient despite continuously suppressed HPV mRNA levels. When treatment with E6 siRNA was coupled with chemotherapy, the p53 activity after treatment with carboplatin and paclitaxel was additively increased, whereas the p53 activation induced by the rest of the drugs was synergistically increased. Treatment with E6 siRNA alone moderately inhibited HeLa cell proliferation, but did not induce detectable apoptosis. The combined cytotoxic effect of E6 siRNA and chemotherapy ranged from subadditive to synergistic, depending on the drug. The decrease of E6 mRNA sensitized HeLa cells for example to doxorubicin and gemcitabine, but counteracted the cytotoxicity of cisplatin and etoposide. In conclusion, activating p53 by degrading E6 mRNA may either increase or decrease the chemosensitivity of cervical cancer cells, depending on the chemotherapy compound.

Introduction

The pathogenesis of cervical cancer is tightly linked to persistent HPV infection (zur Hausen, 2000). High-risk HPV (e.g. HPV 16, 18, 33, 35, 45) genes are found in almost 100 % of cervical cancer tissue samples (Walboomers et al., 1999). Integration of the viral genome and subsequent expression of two main viral oncogenes, E6 and E7, are considered to be critical steps in the development of this particular cancer. E6 binds to p53 tumor suppressor protein and targets it for ubiquitin-mediated degradation (Munger and Howley, 2002). p53 orchestrates the cellular response to various stress stimuli, for example genotoxic damage by ionizing radiation or chemotherapy drugs. Depending on how extensive the damage is, activation of p53 may result in cell cycle arrest, activation of the DNA repair machinery or apoptosis (Vousden and Lu, 2002). Nonfunctional or absent p53 allows accelerated cell division rate and promotes genetic instability, facilitating malignant transformation (Attardi, 2005).

Cervical cancers, even recurrent, rarely carry mutated p53 gene (Lee et al., 1994; Denk et al., 2001), but the p53 protein is constantly targeted for degradation by HPV E6 protein. Because the p53 gene is not altered, different strategies aiming to reactivate the p53 pathway have been vigorously studied. p53 can be activated in cervical cancer cells with some cytotoxic drugs, γ -irradiation, small molecules, by the expression of E2 mRNA and by peptides targeting E6 mRNA, leading to cell senescence, growth inhibition or apoptosis (Butz et al., 2000; Goodwin and DiMaio, 2000; Hietanen et al., 2000; Koivusalo et al., 2002). The strategies based on specific targeting of HPV E6 seem particularly interesting, because these treatments, in theory, affect cervical cancer cells only.

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The introduction of short interfering RNA (siRNA) technology has made it possible to achieve specific, efficient and sustained degradation of the desired target mRNA in mammalian cells (Elbashir et al., 2001). Double stranded RNA (dsRNA) induces degradation of homologous mRNA in a process known as RNA interference (RNAi). In RNAi, long dsRNA is degraded to approximately 22-nt RNA duplexes by the RNase III enzyme Dicer (Bernstein et al., 2001). The resulting effector molecules called short interfering RNA are involved in the sequence-specific targeting and subsequent cleavage of the corresponding target mRNA as part of the RNA induced silencing complex (RISC) (Hammond et al., 2000). SiRNA molecules can efficiently reduce the amount of HPV E6 mRNA in cervical cancer cells, leading to reactivation of p53. The p53 activation was found to inhibit cell growth without inducing marked apoptosis (Jiang and Milner, 2002; Yoshinouchi et al., 2003), in contrast to the study by Butz et al. where the main outcome was apoptosis (Butz et al., 2003).

As current chemotherapy drugs alone are rather inefficient against advanced cervical cancer, new therapeutical approaches are needed. It is possible that the successful targeting of HPV E6 would be beneficial for the treatment response. The effect of activating p53 with E6 siRNA before chemotherapy of cervical cancer cells is not known. In theory, the siRNA-induced p53 activation could specifically sensitize these cells to chemotherapy drugs. However, the role of p53 in response to cancer chemotherapy is far from straightforward (Pirolo et al., 2000; El Deiry, 2003). Abrogation of p53 function protects cervical cancer cells from the cytotoxic effects of several chemotherapy drugs, but increases the cytotoxicity of cisplatin and etoposide (Koivusalo et al., 2002; Koivusalo and Hietanen, 2004). Therefore, before E6 siRNA and chemotherapy are combined *in vivo*, it is of central importance to understand how targeting of E6 mRNA affects the cytotoxicity of different chemotherapy drugs in cervical cancer cells.

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Here, we studied the effects of transfecting HPV 18 positive HeLa cervical cancer cells with siRNA targeting HPV 18 E6 or E7 mRNA, and the outcome of targeting E6 mRNA before chemotherapy with 10 different chemotherapy compounds. The present study shows that both HPV 18 E6 and E7 siRNAs cause only a transient increase in p53 activity in HeLa cells despite continuous suppression of HPV mRNA levels. Sustained and enhanced p53 activation is achieved when treatment with siRNA is coupled with chemotherapy. Most importantly, the degradation of E6 mRNA either enhances or counteracts the cytotoxic effects of concurrent chemotherapy, depending on the drug.

Materials and methods

Cell lines and plasmids.

The HPV 18 positive cervical cancer cell line HeLa was obtained from the ATCC (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (Euroclone, Wetherby, UK) containing 10 % FBS, 2 mM l-glutamine, 1x non-essential amino acids (Euroclone) and 50 µg/ml gentamycin (Calbiochem, San Diego, CA). The HeLa p53 reporter cell line, carrying the p53 reporter plasmid ptkGC₃p53-luc (Lipinski et al., 2001), has been described previously (Koivusalo et al., 2002).

Short interfering RNAs and transfections.

The siRNAs used in this study (Table 1) were designed using HPV 16 and HPV 18 sequence data (NCBI GenBank accession numbers NC_001526 and NC_001357, respectively). The siRNAs were targeted against HPV 16 E6 mRNA (16E6-191 siRNA), HPV 18 E6 mRNA (18E6-165, 18E6-340 and 18E6-385 siRNAs) and HPV 18 E7 mRNA (18E7-604 and 18E7-694 siRNAs). The target sequence of 18E6-165 siRNA is shared by both E6 and E6* transcripts, whereas the target sequences of 18E6-385 and 18E6-340 siRNAs are present only in the unspliced E6/E7 mRNA (Table 1). In the course of the study a paper was published where a vector-derived siRNA was directed against the same sequence as the present 18E6-385 siRNA (Butz et al., 2003). None of the siRNAs is homologous to any known expressed human gene sequence according to the BLAST search. 18E6-340 and 18E6-385 siRNAs were purchased from Dharmacon (Lafayette, CO), the others from ProLigo (Boulder, CO). The delivery of the siRNAs was carried out using OligofectAMINE (Invitrogen, Carlsbad, CA) transfection reagent. SiRNA stock solution was first diluted with annealing buffer

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(Xeragon, Germantown, MD) to a 10 μ M working solution. Consequent steps were performed using Opti-MEM I (Gibco/Invitrogen), OligofectAMINE and DMEM without supplements (Euroclone) as instructed by the manufacturer. The final siRNA concentration in the cell culture medium was 10 nM, 30 nM or 100 nM.

Chemotherapy compounds.

Based on our previous study, in which we analyzed 30 clinically important cytotoxic drugs in attempt to find reactivators of p53 in cervical cancer cells (Koivusalo and Hietanen, 2004), we selected 10 chemotherapy drugs to be used in the present study. Mitomycin (Orion Pharma, Espoo, Finland), oxaliplatin (Sanofi-Synthelabo, Paris, France) and topotecan (GlaxoSmithKline, Uxbridge, Middlesex, UK) were dissolved in sterile water to produce stock solutions. Carboplatin (Bristol-Myers Squibb, Princeton, NJ), cisplatin (Bristol-Myers Squibb) and gemcitabine (a gift from Eli Lilly, Indianapolis, IND) were dissolved in sterile 0.9% NaCl solution. Doxorubicin (Nycomed, Roskilde, Denmark), etoposide (Pfizer, New York, NY), mitoxantrone (Wyeth-Lederle Finland, Vantaa, Finland) and paclitaxel (Bristol-Myers Squibb) were ready infusion concentrates/solutions or included its own infusion/injection solution. The stock solutions were stored at -20 C° and were diluted in DMEM immediately before use.

p53 reporter assay.

The ptkGC₃p53luc-bsd HeLa p53 reporter cells (Koivusalo et al., 2002; Lipinski et al., 2001) were seeded in triplicate into 96-well plates (4×10^3 cells/well) and transfected with siRNA 24 h later. The cells were then either incubated with siRNA alone, or were additionally treated with chemotherapy drugs. After incubation, the number of living cells in each well

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was estimated using the colorimetric WST-1 assay (Roche, Mannheim, Germany). The luciferase activity was determined with the Bright-GloTM luciferase assay reagent (Promega, Madison, WI). In both assays, the plates were read in the Wallac Victor² microplate reader (Wallac Oy, Turku, Finland). The p53 readings were normalized against the WST-1 values.

Clonogenic assay.

When the combined cytotoxic effect of siRNA and chemotherapy was studied, HeLa cells were plated in triplicate in 96-well plates (4×10^3 cells/well) and were transfected 24 h later with 100 nM siRNA. After 24 h incubation with siRNA, drugs or medium alone were added for 6 h. The cells from triplicate wells were then trypsinized, pooled, and re-plated in triplicate to 6-well plates. After having formed clearly visible clones (7 days), the cells were fixed with 1:1 acetone/methanol and stained with Giemsa (Merck, Whitehouse Station, NJ). The plates were scanned and the resulting images were analyzed with Adobe Photoshop 5.0 software (Adobe, San Jose, CA). The threshold adjustment function was used to produce a binary image. The total pixel count of each well was determined with histogram analysis. The survival fractions were obtained by comparing pixel counts from treated wells to pixel counts from non-treated wells. The experiments involving HeLa CMV and HeLa DD cells were performed as described above, except that the cells were plated for clonogenic growth after 24 h incubation with siRNA, and that the clones were also counted manually.

Quantitative real-time PCR (qRT-PCR).

The method has been described earlier (Koivusalo et al., 2002). Briefly, HeLa cells were plated in 12-well plates (40×10^3 cells/well) and were transfected with siRNA 24 h later. RNA was extracted with RNeasy mini kit (Qiagen, Hilden, Germany). Extracted RNA was

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treated with DNase I (Gibco) before performing first strand cDNA synthesis (Applied Biosystems, Foster City, CA). The amplifications were carried out with GeneAmp 5700 sequence detection system using SYBR green PCR master mix (Applied Biosystems). The primers used were: HPV 18 E6; forward: 5'-TGGCGCGCTTTGAGGA-3' reverse: 5'-TGTTCAAGTTCGGTGCACAGATC-3', HPV 18 E7; forward: 5'-TGTATTGCATTTAGAGCCCAAA-3' reverse: 5'-CTTCCTCTGAGTCGCTTAATTGC-3' EF1 α ; forward: 5'-CTGAACCATCCAGGCCAAAT-3' reverse: 5'-GCCGTGTGGCAATCCAAT-3'. Each experiment was performed in triplicate. The relative E6 and E7 mRNA expression levels were calculated by adjusting the E6 and E7 readings against the readings obtained for the housekeeping gene EF1 α .

Immunofluorescence microscopy.

HeLa cells were first plated in 8-well LabTekTM chamber slides (Nalge Nunc, Rochester, NY) at a density of 8×10^3 cells/well. After 24 h, the cells were transfected with 30 nM siRNA. Then, the cells were either incubated with siRNA alone or were additionally treated with chemotherapy drugs. The cells were fixed at 4 °C with 1:1 acetone/methanol. Detection of p53 protein was carried out with the monoclonal p53 antibody DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and a TRITC conjugated goat anti-mouse IgG secondary antibody (Zymed, South San Francisco, CA). DNA staining dye Hoechst 33258 (Calbiochem) was used to visualize the nuclei. The stained cells were examined with Olympus BX60 microscope (Olympus, Melville, NY) and the fluorescent images were acquired with analySIS 4.17 software (Soft Imaging System GmbH, Münster, Germany).

Statistical analysis.

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The differences between the mean values of surviving HeLa cell clones and between the mean values of average clone sizes in Fig. 4 were statistically tested with one-way analysis of variance (ANOVA). After performing ANOVA the post-hoc comparisons between each treatment and the control were carried out using Dunnett's multiple comparison method. In the analysis of synergism, a nonlinear dose-response curve with 95 % confidence intervals was calculated for each siRNA + drug combination. Three forms of functions were applied to fit the data: sigmoid median-effect dose-response relation, third order polynomial-effect dose-response relation and logistic-effect dose-response relation. The best fitting form of function was the function with the smallest mean square error. The fit was also graphically confirmed. The theoretical zero-interaction (exactly additive) dose-response curve for each siRNA + drug combination was calculated by applying Bliss independence criterion (Suhnel, 1996; Tallarida, 2000) The statistical evaluation of the synergism was done by comparing each siRNA + drug dose-response curve with the 95 % confidence intervals to the respective Bliss' independence curve.

Results

Transfecting HeLa cells with siRNA targeting HPV 18 E6 or E7 mRNA: effect on HPV mRNA levels, p53 activity and cell viability.

It has been shown that HPV mRNA can be degraded with siRNA, but the data presented by these studies are controversial. It has been reported that HPV 16 E6 and E7 mRNA are selectively degraded when the respective mRNA is targeted (Jiang and Milner, 2002). In contrast, two other studies conclude that selective degradation of E6 mRNA is possible only by targeting the sequence between the E6* splice sites, and that targeting of E7 mRNA should decrease the amount of both E6 and E7 mRNA (Butz et al., 2003; Yoshinouchi et al., 2003). Because of these controversial observations we first studied the consequences of targeting different HPV 18 mRNA sequences with synthetic siRNAs (Table 1). When E7 mRNA or a sequence common to both E6 and E6* transcripts was targeted, both E6 and E7 mRNA levels were equally decreased by 50-80 % (Fig. 1). In contrast, targeting the sequence between HPV 18 E6* splice donor-acceptor sites (Schneider-Gadicke et al., 1988) with 18E6-385 siRNA induced selective degradation of E6 mRNA (Fig. 1). With each siRNA, the decrease of HPV mRNA was detected already at 12 h after transfection, and the effect lasted for at least 96 h (Fig. 1). The 16E6-191 siRNA, that does not have a target in HeLa cells, and 18E6-340 siRNA did not show degradation of HPV 18 mRNA (data not shown), but were utilized in the study as inactive reference siRNAs. None of the present siRNAs was able to reduce the amount of ABL, β -actin or GUS mRNA in HeLa cells, indicating that these siRNAs do not cause generalized degradation of cellular mRNA (supplemental data).

In experiments performed with HeLa p53 reporter cells, each functional HPV 18 siRNA induced a ~4-fold activation of the p53 reporter compared to the reference siRNA 16E6-191,

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the peak values being measured at 72 h after transfection (Fig. 2). Surprisingly however, the p53 reporter activities repeatedly returned close to control levels within the following 24 h (Fig. 2). To exclude the possible p53-activating effect of 16E6-191 siRNA, we performed additional time course experiments with HeLa p53 reporter cells. 16E6-191 siRNA was found to cause a marginal (~20 %) but constant elevation in the p53 reporter baseline activity, probably through nonspecific stress (supplemental data). In immunofluorescence microscopy experiments, modest p53 staining was observed in some of the HeLa cells that were transfected 12 h earlier with 18E6-385 siRNA, but at 48 h, strong nuclear p53 staining was detected in most cells (Fig. 3). At 96 h only weakly stained cells were seen despite the continuously suppressed HPV mRNA levels (Fig. 3). Death of p53-positive cells could explain the disappearance of strongly staining cells, but no signs of apoptosis were detected with the nuclear dye Hoechst 33258 (Fig. 3). The p53 staining of cells treated with the nonhomologous siRNA 16E6-191 did not differ from the p53 staining of non-treated cells at any time point (Fig. 3). The p53 staining after transfection with 18E6-165, 18E7-604 and 18E7-694 siRNA was at each time point essentially identical to the staining observed with 18E6-385 siRNA (data not shown).

In the p53 reporter experiments HeLa cell survival was reduced by only ~20 % after 96 h incubation with the functional HPV 18 siRNAs (data not shown). The morphology of the transfected cells was examined daily with light microscopy, but no obvious indications of apoptosis were seen after transfection with any siRNA (data not shown). To elucidate the role of p53 in the siRNA-induced growth suppression, we performed clonogenic experiments using HeLa CMV and HeLa DD cell lines. HeLa DD cells are stably transfected with a plasmid that expresses a truncated dominant negative p53 protein and therefore abrogates wild-type p53 function, whereas HeLa CMV cells carry the empty vector. The selective degradation of E6 mRNA by 18E6-385 siRNA decreased the number of surviving HeLa

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CMV cell clones by only $20 \pm 7\%$ (SD), but the final average clone size was clearly restricted, being $42 \pm 8\%$ of the size of non-treated HeLa CMV cell clones (Fig. 4). In contrast, the average size of HeLa DD cell clones after treatment with 18E6-385 siRNA was $73 \pm 10\%$ of the size of non-treated HeLa DD cell clones, and the number of surviving clones was reduced by $12 \pm 11\%$ (Fig. 4). Therefore, approximately half of the growth inhibitory effect of 18E6-385 siRNA can be explained by p53-dependent mechanisms. The inactive reference siRNA 18E6-340 diminished the amount of surviving HeLa CMV and HeLa DD cell clones by $10 \pm 6\%$ and $7 \pm 9\%$, respectively, and the final average clone sizes were $79 \pm 11\%$ and $90 \pm 7\%$ in comparison to non-treated clones (Fig. 4).

p53 activity in HeLa cells after concurrent treatment with E6 siRNA and chemotherapy.

Several chemotherapy drugs can activate p53 in cervical cancer cells despite the presence of HPV E6. We studied the outcome of transfecting HeLa p53 reporter cells with E6 siRNA before chemotherapy with 10 different chemotherapy drugs. Of these 10 drugs the following 6 were selected because they are potent p53 activators in cervical cancer cells: the anthracyclin doxorubicin, the anthracyclin-related drug mitoxantrone, the antibiotic mitomycin, the nucleoside analogue gemcitabine, the topoisomerase I inhibitor topotecan and the topoisomerase II inhibitor etoposide (Koivusalo and Hietanen, 2004). The platinum derivatives carboplatin, cisplatin and oxaliplatin were included because of the central role of cisplatin in the chemotherapy of cervical cancer. The microtubule inhibitor paclitaxel, which causes only negligible p53 activation in cervical cancer cells, was selected for comparative purposes.

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Chemotherapy alone induced a 1-8 fold activation of the p53 reporter, depending on the drug (Fig. 5A). Whereas only a transient increase in p53 activity was seen after treatment with siRNA alone, sustained and synergistically increased p53 activation was detected when the selective degradation of E6 mRNA by 18E6-385 siRNA was coupled with chemotherapy (Fig. 5A). Two drugs, carboplatin and paclitaxel, produced only an additive effect in combination with 18E6-385 siRNA (Fig. 5A). To see whether the degradation of E7 mRNA affects drug-induced p53 activation, we transfected HeLa cells with 18E6-165 siRNA, which degrades both E6 and E7 mRNA, and then treated the cells with doxorubicin, etoposide or gemcitabine. The resulting p53 reporter activities were very similar to those seen after concurrent treatment with 18E6-385 siRNA and the respective chemotherapy compounds (data not shown). Therefore, additional degradation of E7 mRNA does not alter the p53 activation induced by doxorubicin, etoposide or gemcitabine. The inactive reference siRNA 18E6-340 did not enhance the chemotherapy-induced p53 reporter activation (data not shown).

The p53 reporter data is in line with the p53 staining in immunofluorescence microscopy experiments. Treatment with 18E6-385 siRNA in combination with doxorubicin, etoposide or gemcitabine produced a p53 staining pattern where both moderately and intensely staining nuclei were observed, whereas only moderately staining nuclei were detected after treatment with chemotherapy alone (Fig. 6). At this time point, 96 h after transfection, the p53 staining in cells treated with siRNA alone was rather weak (Fig. 6). Transfecting HeLa cells with the nonhomologous 16E6-191 siRNA did not enhance the p53 staining induced by doxorubicin, etoposide or gemcitabine (Fig. 6). When both E6 and E7 mRNA were degraded with 18E6-165 siRNA before chemotherapy with doxorubicin, etoposide and gemcitabine, the resulting p53 staining patterns were essentially identical to those seen when E6 mRNA was selectively degraded with 18E6-385 siRNA before treatment with the respective drugs (data not shown).

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These data demonstrate that it is possible to significantly enhance chemotherapy-induced p53 activation by targeting E6 mRNA before chemotherapy, and that siRNA-mediated degradation of E6 mRNA does not lead to sustained p53 activation in HeLa cells unless there is a simultaneous proper stimulus for p53, for example DNA-damage by chemotherapy drugs.

The combined cytotoxic effect of E6 siRNA and chemotherapy.

To evaluate the combined effect of siRNA and chemotherapy on acute cell death, we analyzed the cell survival data from p53 reporter experiments using statistical modelling. A clear synergistic cytotoxic effect was observed when 18E6-385 siRNA was combined with doxorubicin, gemcitabine, mitomycin, mitoxantrone, paclitaxel and topotecan (Fig. 5B). In contrast, the combined cytotoxic effect of 18E6-385 siRNA and etoposide was subadditive, whereas the cytotoxicity of the rest of the drugs was additively increased when combined with 18E6-385 siRNA (Fig. 5B). To study longer-term sequelae of targeting E6 before chemotherapy we performed clonogenic cell survival experiments with HeLa cells. Again, the combined cytotoxic effect of 18E6-385 siRNA and chemotherapy ranged from subadditive to synergistic, depending on the drug. Chemotherapy drugs that produced a synergistic cytotoxic effect in combination with 18E6-385 siRNA were doxorubicin, gemcitabine, mitoxantrone, paclitaxel and topotecan (Fig. 7). The cytotoxicity of carboplatin, mitomycin and oxaliplatin was additively increased when the cells were transfected with 18E6-385 siRNA (Fig. 7). In contrast to the other drugs, cisplatin and etoposide caused a subadditive cytotoxic effect in combination with 18E6-385 siRNA (Fig. 7). Thus, HeLa cells became more resistant to etoposide and especially to cisplatin after E6 mRNA levels were reduced with 18E6-385 siRNA. Transfecting HeLa cells with the reference siRNA 18E6-340 did not alter chemotherapy-induced cytotoxicity with the exception of the slight right shift seen in the dose-response curve of mitomycin (supplemental data). Moreover, incubating

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HeLa cells with the siRNA transfection reagent for 96 h did not affect cell viability (supplemental data). These findings indicate that the degradation of E6 mRNA, and not the siRNA transfection *per se*, is responsible for modulating drug-induced cytotoxicity.

Despite being statistically significant, most of the changes in clonogenic cell survival rates after combining 18E6-385 siRNA to chemotherapy are rather small. However these changes have a clear tendency to occur in the same direction in both short and long term cytotoxicity assays. This suggests that same biological mechanisms are operational regardless of the drug concentration or the experimental setting. With most drugs the synergistic effects with siRNA appear to be more pronounced in the short term cytotoxicity assay than in clonogenic experiments. One explanation is that 18E6-385 siRNA not only sensitizes HeLa cells to e.g. mitoxantrone but also accelerates mitoxantrone-induced cell death by increasing p53 activity. Nevertheless, the cytotoxicity assays show that it is possible to sensitize HeLa cells to several clinically relevant chemotherapy drugs by targeting HPV 18 E6 mRNA with siRNA. However some drugs appear to be less cytotoxic in HeLa cells when E6 mRNA is degraded with siRNA before chemotherapy.

Discussion

Means of targeting HPV E6 for therapeutic gain have been actively studied, and currently the siRNA-technology appears to be the most promising method. However, the present data argue against the possibility that treatment with siRNA alone would efficiently kill cervical cancer cells in human patients. None of the present synthetic HPV 18 siRNAs, of which two were targeted against E6 and two against E7 mRNA, was able to initiate apoptosis in HeLa cells. Furthermore, the siRNA-induced p53 activation faded quickly unless the cells were additionally treated with chemotherapy compounds.

HPV E6 and E7 mRNA are derived from several bicistronic E6/E7 mRNA species, which contain either the complete E6 mRNA or a truncated form of E6 mRNA (E6* mRNA) in addition to E7 mRNA (Schwarz et al., 1985; Schneider-Gadicke and Schwarz, 1986). In the present study targeting of E7 mRNA or a sequence common to both E6 and E6* transcripts resulted in the degradation of both E6 and E7 mRNA in HeLa cells, which is explained by the bicistronic nature of HPV 18 E6/E7 transcripts. In contrast, targeting the sequence between E6* splice donor-acceptor sites led to selective E6 mRNA degradation, because only the full length E6/E7 mRNA was affected. The present results are in line with a study where selective degradation of HPV 18 E6 mRNA was achieved by targeting a sequence between E6* splice sites, and with another study where targeting a sequence shared by both HPV 16 E6 and E6* mRNA resulted in the decrease of both E6 and E7 mRNA (Butz et al., 2003; Yoshinouchi et al., 2003), but are in contrast with a study where targeting of HPV 16 E7 mRNA induced the selective degradation of E7 mRNA in CaSki and SiHa cells (Jiang and Milner, 2002). The selective targeting of HPV E7 mRNA is difficult to reconcile with the present results, and with the fact that E7 mRNA of both HPV 16 and HPV 18 is unspliced and stems from bicistronic E6/E7 mRNA species.

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In clonogenic cell survival experiments the 18E6-385 siRNA-mediated degradation of E6 mRNA produced a moderate growth inhibitory effect, of which approximately half was blocked when p53 function was abrogated with a dominant negative p53. Therefore half of the 18E6-385 siRNA-induced growth suppression is caused by p53-independent mechanisms. These may include e.g. the release of other cellular targets than p53 from E6 binding, nonspecific toxicity, or both. Since the inactive reference siRNA 18E6-340 also slightly reduced HeLa cell survival, regardless of the p53 status, some or even all HPV siRNAs may induce nonspecific effects. Transfection with siRNA can activate stress response genes, e.g. GADD and p38, and there is evidence that siRNA might degrade off-target mRNAs in human cells (Semizarov et al., 2003; Jackson et al., 2003). Nonspecific effects may partially explain why cytotoxicity can occur after transfection with seemingly nonfunctional siRNAs, and underline the importance of siRNA optimization.

The transient nature of the siRNA-induced p53 activation may at least partially explain the lack of apoptosis after treatment with siRNA alone. In addition, nonfunctional p53 allows the accumulation of growth-promoting and anti-apoptotic mutations, which probably make cervical cancer cells less dependent on HPV oncogenes over time. The present results are in line with two other studies, where growth inhibition was the main outcome after transfecting cervical cancer cells with HPV siRNAs (Jiang and Milner, 2002; Yoshinouchi et al., 2003). However, in one study massive apoptosis and almost complete inhibition of clonogenic cell growth occurred after HeLa cells were transfected with a vector that expressed a siRNA targeting the same sequence as the present synthetic 18E6-385 siRNA (Butz et al., 2003). The stronger effect seen in this study may be partially explained by the constant production of new siRNA molecules by the vector, the more efficient degradation of E6 mRNA (80% versus 50% decrease) and the selection of transfected cells with neomycin. Due to incomplete

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transfection efficiency a proportion of cells remained untransfected in our experiments, which might decrease the signal. However the authors report that even their synthetic siRNA induced dramatic apoptosis. This siRNA targeted different nucleotides (381-399) than their vector-derived siRNA and any of the siRNAs in the present study. Since we saw only transient accumulation of nuclear p53 in HeLa cells after transfection with E6 and E7 siRNAs despite continuously suppressed HPV mRNA levels, it is more likely that p53 is cleared from the nucleus if the culture conditions do not evoke additional stress that could trigger p53-mediated apoptosis. The specific nature of the p53-antagonizing mechanism is not yet known, but the strongly enhanced p53 activation after concurrent treatment with siRNA and chemotherapy suggests that this mechanism is inactive when low E6 mRNA levels are coupled with a proper stimulus for p53, for example DNA-damage by cytotoxic drugs. This is an important finding considering the possible use of siRNA in cervical cancer therapy, as it questions the use of HPV siRNA as a monotherapeutic agent.

The siRNA-mediated degradation of E6 mRNA had variable effect on chemotherapy-induced p53 activation: their combined effect ranged from additive to synergistic, depending on the drug. We have recently shown that out of 30 cytotoxic drugs, nearly all reduce the amount of E6 mRNA but only some activate p53 in SiHa cervical cancer cells (Koivusalo and Hietanen, 2004). In that study the magnitude of p53 activation did not correlate with E6 mRNA levels. In the present study, carboplatin and paclitaxel caused only additive p53 activation with siRNA, indicating that not all drugs produce strong p53 activation when coupled with E6 siRNA. It is unlikely that the strongly enhanced p53 activation after concurrent treatment with siRNA and chemotherapy is the direct result of decreased E6 mRNA levels. Rather, it appears that the decrease of E6 mRNA permits enhanced p53 activation under right circumstances, e.g. in the presence of a cytotoxic drug that can generate p53-activating signals. The degradation of E7 mRNA does not seem to modulate chemotherapy-induced p53

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activation, because essentially identical p53 activities were measured after chemotherapy with doxorubicin, etoposide and gemcitabine regardless of whether only E6 mRNA or both E6 and E7 mRNA were degraded with siRNA before chemotherapy.

Although the chemotherapy-induced p53 activation can be enhanced with siRNA, it is not self-evident that this will actually lead to increased cytotoxicity. We have previously shown that disruption of p53 function partially protects SiHa cells from the cytotoxic effects of carboplatin, doxorubicin, gemcitabine, mitoxantrone and oxaliplatin, but increases the cytotoxicity of cisplatin and etoposide, whereas the cytotoxicity of mitomycin, paclitaxel and topotecan does not depend on the p53 status (Koivusalo et al., 2002; Koivusalo and Hietanen, 2004). In the present study, the 18E6-385 siRNA-mediated degradation of E6 mRNA synergistically increased the cytotoxicity of doxorubicin, gemcitabine, mitoxantrone paclitaxel and topotecan in the clonogenic cell survival experiments. The cytotoxic effect of carboplatin, mitomycin and oxaliplatin was additively increased when combined with 18E6-385 siRNA. In contrast, transfection with 18E6-385 siRNA rendered HeLa cells more resistant to cisplatin and etoposide. Because p53 is a key regulator of DNA repair proteins (Adimoolam and Ford, 2003), it is possible that the enhanced p53 activation by E6 siRNA might actually increase resistance to some drugs by promoting the repair of damaged DNA. Although all platinum derivatives form adducts with DNA, the role of different DNA repair effectors is not uniform in response to different platinum compounds, which may partially explain the varying effect of active p53 on platinum drug-induced cytotoxicity. For example, defective mismatch repair provides resistance to cisplatin but not to oxaliplatin (Fink et al., 1996). The result that 18E6-385 siRNA increases resistance to cisplatin is consistent with our previous studies. However, at first glance it seems surprising that 18E6-385 siRNA only additively increased the cytotoxicity of carboplatin and oxaliplatin although these drugs are less cytotoxic when p53 function is abrogated. Nevertheless, 18E6-385 siRNA increased the

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p53 activation by carboplatin and oxaliplatin only modestly. It is possible that the p53 activation induced by these drugs alone is sufficient to cause maximal amount of p53-dependent cytotoxicity which cannot be further enhanced by increasing p53 activity with E6 silencing. Interestingly, 18E6-385 siRNA sensitized HeLa cells to paclitaxel. In our previous study the expression of ectopic E6 protected SiHa cells from the cytotoxic effects of paclitaxel, whereas rendering p53 nonfunctional with a dominant negative p53 did not affect the potency of paclitaxel (Koivusalo and Hietanen, 2004). Besides p53, HPV 18 E6 is known to target other cellular proteins, for example the proapoptotic factor Bak (Thomas and Banks, 1998). Therefore, siRNA-mediated degradation of E6 mRNA probably regulates sensitivity to cytotoxic drugs by several distinct mechanisms.

The present study shows that chemotherapy-induced p53 activation and cytotoxicity can be enhanced in HeLa cells by pre-treating them with E6 siRNA, and suggests that RNA interference has therapeutic potential in the treatment of cervical cancer when combined with chemotherapy. The present results also stress the importance of knowing how different chemotherapy drugs work in combination with siRNA. HPV E6 siRNA may have therapeutic potential as a chemosensitizer of cervical cancer cells, but only in combination with certain drugs.

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Footnotes.

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Legends for figures

Fig. 1. HPV 18 E6 and E7 mRNA levels in HeLa cells after treatment with E6 and E7 siRNAs. HeLa cells were transfected with siRNA targeting HPV 16 E6, HPV 18 E6 or HPV 18 E7 mRNA. The cells were incubated with siRNA for 12, 48 or 96 hours before extracting RNA and determining the expression levels of E6, E7 and the housekeeping gene EF1 α with quantitative real-time PCR. Relative E6 and E7 mRNA levels were obtained by normalizing the E6 and E7 values against EF1 α values. —▲—, 10 nM siRNA. —○—, 30 nM siRNA. —●—, 100 nM siRNA. Data points represent mean values from triplicate samples, error bars are \pm SD.

Fig. 2. Activation of the p53 reporter after transfecting HeLa p53 reporter cells with siRNA targeting HPV 18 mRNA. After incubation with siRNA, luciferase activity was quantitated and normalized against the relative cell number obtained by colorimetric WST-1 analysis. The p53 reporter activities are expressed as a fold increase to the luciferase reading after transfecting the cells with the control siRNA 16E6-191, which has no target in HPV 18 positive HeLa cells. —▲—, 10 nM siRNA. —○—, 30 nM siRNA. —●—, 100 nM siRNA. Data points represent mean values, error bars are \pm SD from triplicate wells.

Fig. 3. Kinetic changes in the amount of nuclear p53 in HeLa cells after transfection with E6 siRNA. HeLa cells were transfected with 30 nM siRNA targeting either HPV 16 or HPV 18 E6 mRNA. p53 was detected by immunofluorescence microscopy at 12, 48 and 96 h after transfection with siRNA. The detection was carried out with the DO-1 primary antibody and a TRITC-labeled secondary antibody. The nuclei were visualized with Hoechst 33258. The experiment was performed three times, representative pictures are shown.

Fig. 4. Clonogenic survival of HeLa cells after siRNA-mediated degradation of E6 mRNA. HeLa DD cells, expressing a truncated dominant negative p53, and HeLa CMV cells, carrying the empty vector, were plated in triplicate to 96-well plates. After 24 h the cells were transfected with 18E6-385 siRNA (100 nM) or with the inactive reference siRNA 18E6-340 (100 nM), or were treated with medium alone. The cells were incubated with siRNA for 24 h before they were trypsinized, suspended in fresh medium and re-plated in 6-well plates. After 7 days, the resulting colonies were fixed, stained and analyzed. The clones were counted manually, and the total surface area of clones in each well was determined by pixel analysis after the plates were scanned. Relative average clone size was obtained by dividing the pixel count of each well with the number of clones in the respective well, and then comparing the resulting value to the average clone size of nontreated cells. White bars express the relative number of surviving clones, black bars represent the relative average clone size. The bars show mean values \pm 95 % CI from two independent experiments, both performed in triplicate. The groups were statistically compared using ANOVA with Dunnett's post test; ***, $P < 0.001$. The survival rates of HeLa CMV versus HeLa DD cells after transfection with 18E6-385 siRNA were compared by performing two-tailed paired t tests. In both HeLa CMV and DD cells the number of surviving clones decreased significantly less than the average clone size ($P < 0.0001$ and $P = 0.0002$ respectively). The reduction in average HeLa CMV clone size is significantly greater than the reduction in average HeLa DD clone size ($P < 0.0001$), but the mean numbers of surviving HeLa CMV versus HeLa DD clones do not significantly differ ($P = 0.0724$).

Fig. 5. p53 reporter activity and cytotoxicity in HeLa cells after treatment with E6 siRNA and/or chemotherapy drugs. HeLa p53 reporter cells were transfected with 30 nM 18E6-385

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siRNA 48 h before drug treatment. After administering the drugs, the cells were incubated for another 48 h. Then, luciferase activity was quantitated and normalized against the relative cell number obtained by colorimetric WST-1 analysis. Relative p53 reporter activities were acquired by comparing the normalized luciferase readings from treated cells to the readings from nontreated cells. A, □, siRNA alone. —■—, drug alone. —○—, 18E6-385 siRNA + drug. — — —, Bliss independence curve which indicates the theoretical situation where the combined effect of siRNA and chemotherapy is exactly additive. Data points represent mean values from triplicate wells, error bars are \pm SD. B, cell survival data measured from the same wells as the luciferase activities in figure 5A. The data has been statistically modelled as described in Materials and Methods to obtain siRNA + drug dose response curves (—) with 95 % confidence interval limits (·····). — — —, Bliss curve showing the exactly additive situation for each siRNA + drug combination. If the Bliss curve is above the upper 95 % confidence interval limit, the combined effect of siRNA + chemotherapy is considered to be synergistic.

Fig. 6. Nuclear accumulation of p53 in HeLa cells after concurrent treatment with E6 siRNA and chemotherapy drugs. HeLa cells were first transfected with 30 nM siRNA targeting either HPV 16 or HPV 18 E6 mRNA, control cells received medium alone. After 48 h, the cells were treated with 0.5 μ M doxorubicin, 5 μ M etoposide, 50 μ M gemcitabine or medium alone. The p53 protein was detected by immunofluorescence microscopy 48 h after the drug addition, using the DO-1 primary antibody and a TRITC-labeled secondary antibody. Hardly any p53 staining was detected after treatment with 16E6-191 siRNA or medium alone. Some, but not all, cells stained positively for p53 after treatment with 18E6-385 siRNA. Each chemotherapy drug alone induced a moderate nuclear accumulation of p53 in most of the cells. Concurrent treatment with 18E6-385 siRNA and chemotherapy resulted in altered p53 staining pattern: both moderately and intensely staining cells were seen. Some of the

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intensely staining cells are pointed by white arrowheads. In contrast, 16E6-191 siRNA did not enhance the chemotherapy-induced accumulation of p53. Shown are representative views from two independent experiments.

Fig. 7. Clonogenic survival of HeLa cells after treatment with E6 siRNA and/or chemotherapy. The cells were incubated with 100 nM 18E6-385 siRNA for 24 h before adding chemotherapy drugs to the medium. Six hours after the drug addition the cells were trypsinized, suspended in fresh medium and plated in 6-well plates. After 7 days, the resulting colonies were fixed, stained and scanned. The survival fractions were obtained by comparing pixel counts from wells treated with medium only to counts from wells treated with siRNA and/or chemotherapy. A, □, siRNA alone. —■—, drug alone. —○—, 18E6-385 siRNA + drug. Data points represent mean values from triplicate wells, error bars are \pm SD. B, the data presented in figure 7A have been statistically analyzed as described in Materials and Methods to obtain siRNA + drug dose response curves (—) with 95 % confidence interval limits (·····). The theoretical zero-interaction (exactly additive) situation for each siRNA + drug combination was calculated by applying Bliss independence criterion. If the Bliss curve (---) is above the upper 95 % confidence interval limit, the combined effect of siRNA + chemotherapy is considered to be synergistic.

TABLE 1
siRNA composition and target sequence location in HPV genome

siRNA	Duplex Composition	Target Sequence Location	Target Transcript(s)
18E6-165	5'- CUGAACACUUCACUGCAAG dTdT GACUUGUGAAGUGACGUUC -5'	dTdT HPV 18, 165-183 -5'	E6* mRNA, full length E6 mRNA
18E6-340	5'- GACAUUAUUCAGACUCUGU dTdT CUGUAAUAAGUCUGAGACA -5'	dTdT HPV 18, 340-358 -5'	full length E6 mRNA
18E6-385	5'- CUAACACUGGGUUAUACAA dTdT GAUUGUGACCCAAUAUGUU -5'	dTdT HPV 18, 385-403 -5'	full length E6 mRNA
18E7-604	5'- GGCAACAUUGCAAGACAUU dTdT CCGUUGUAACGUUCUGUAA -5'	dTdT HPV 18, 604-622 -5'	E7 mRNA
18E7-694	5'- GGAAGAAAACGAUGAAAUA dTdT CCUUCUUUUGCUACUUUAU -5'	dTdT HPV 18, 694-712 -5'	E7 mRNA
16E6-191	5'- UGUGUGUACUGCAAGCAAC dTdT ACACACAUGACGUUCGUUG -5'	dTdT HPV 16, 191-209 -5'	E6* mRNA, full length E6 mRNA

Figure 1

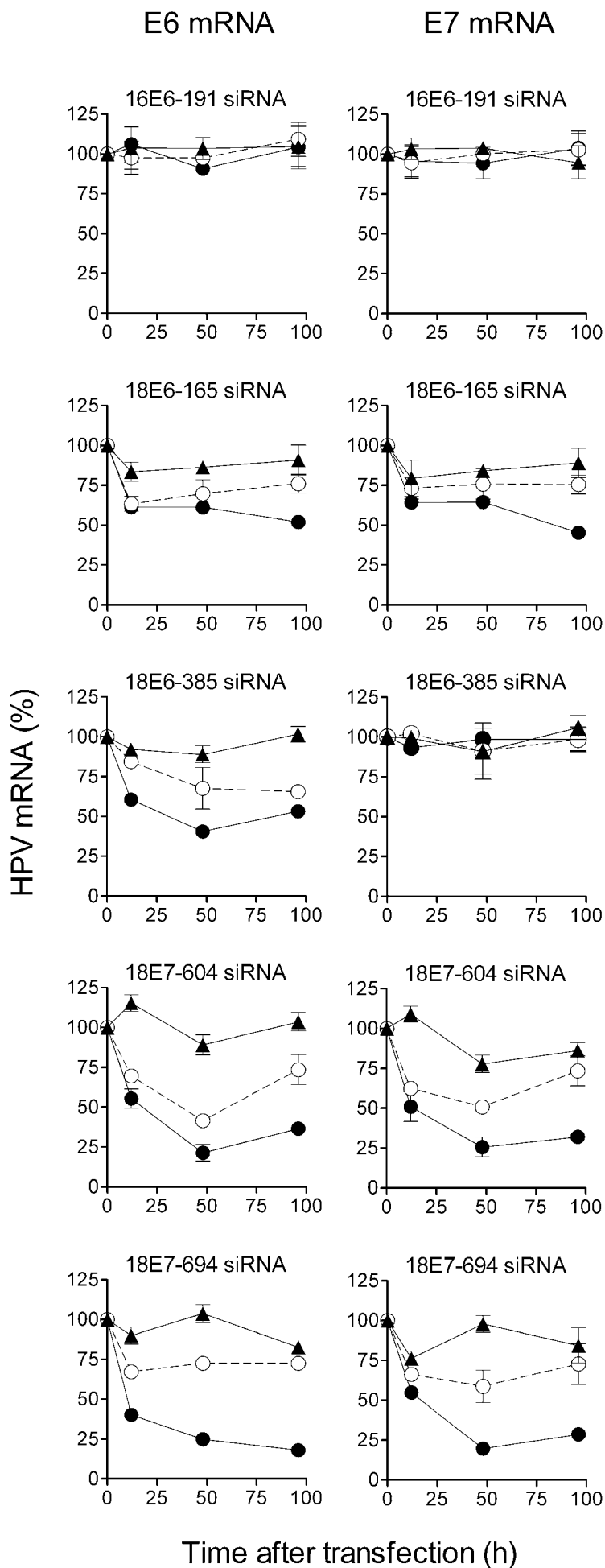


Figure 2

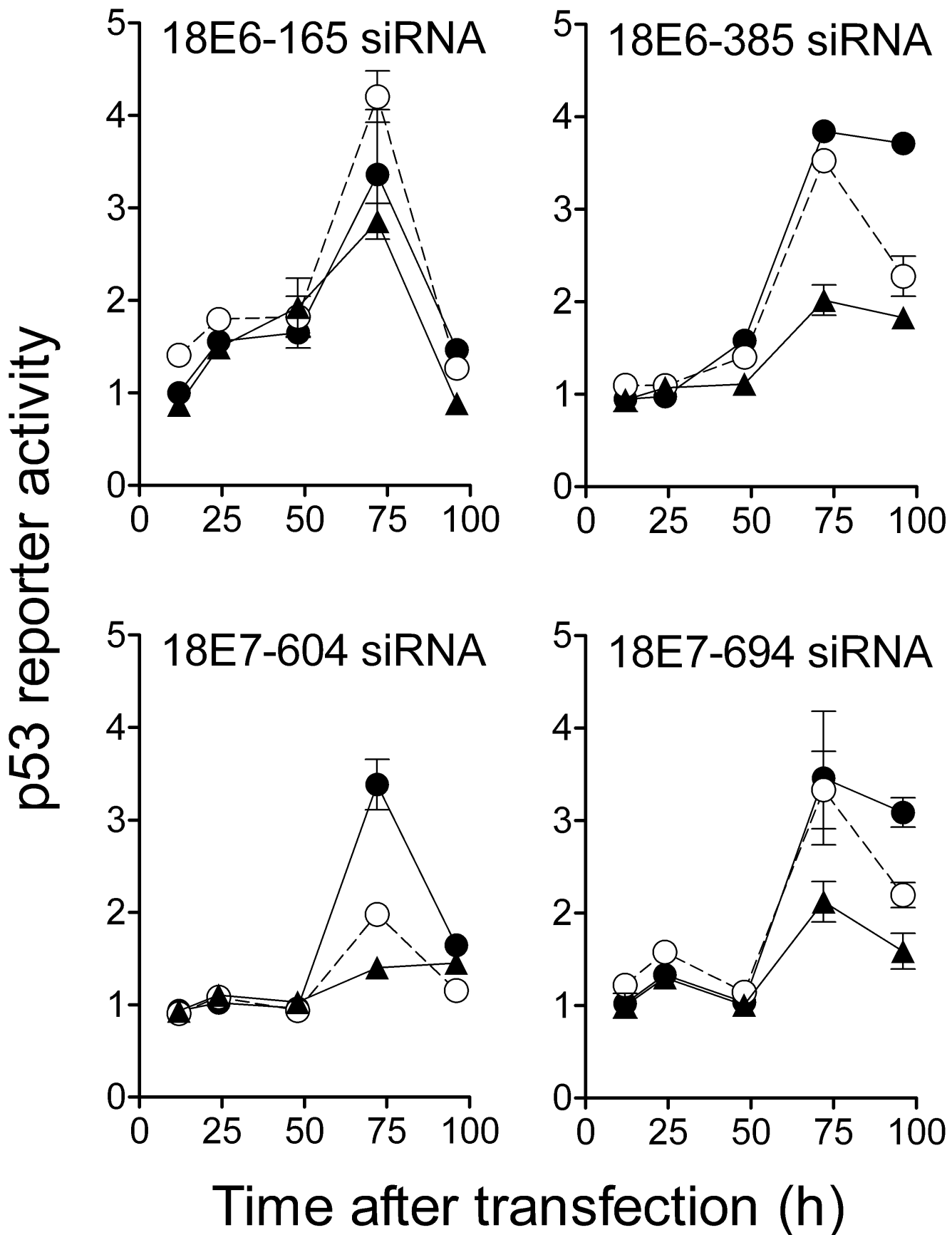


Figure 3

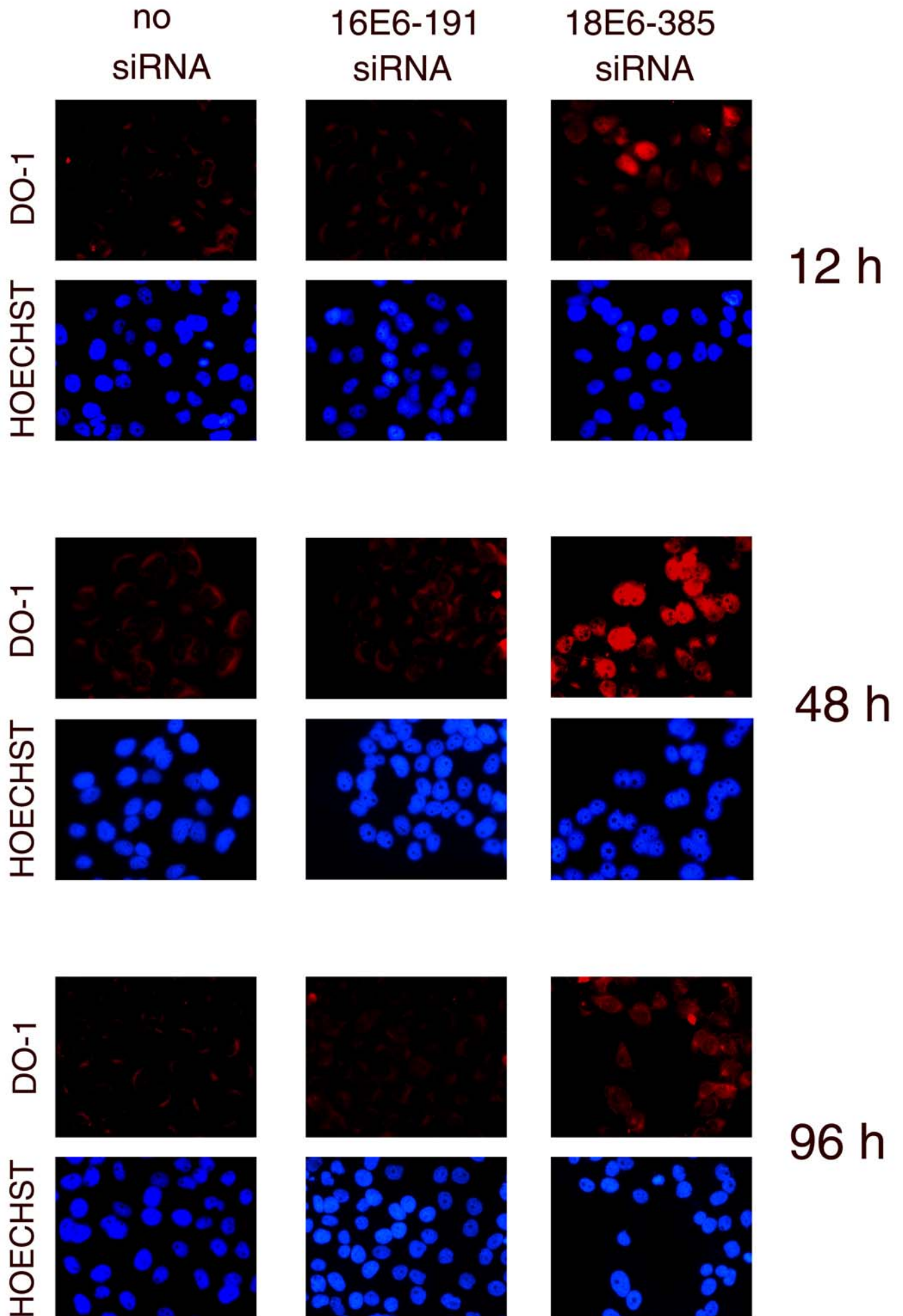


Figure 4

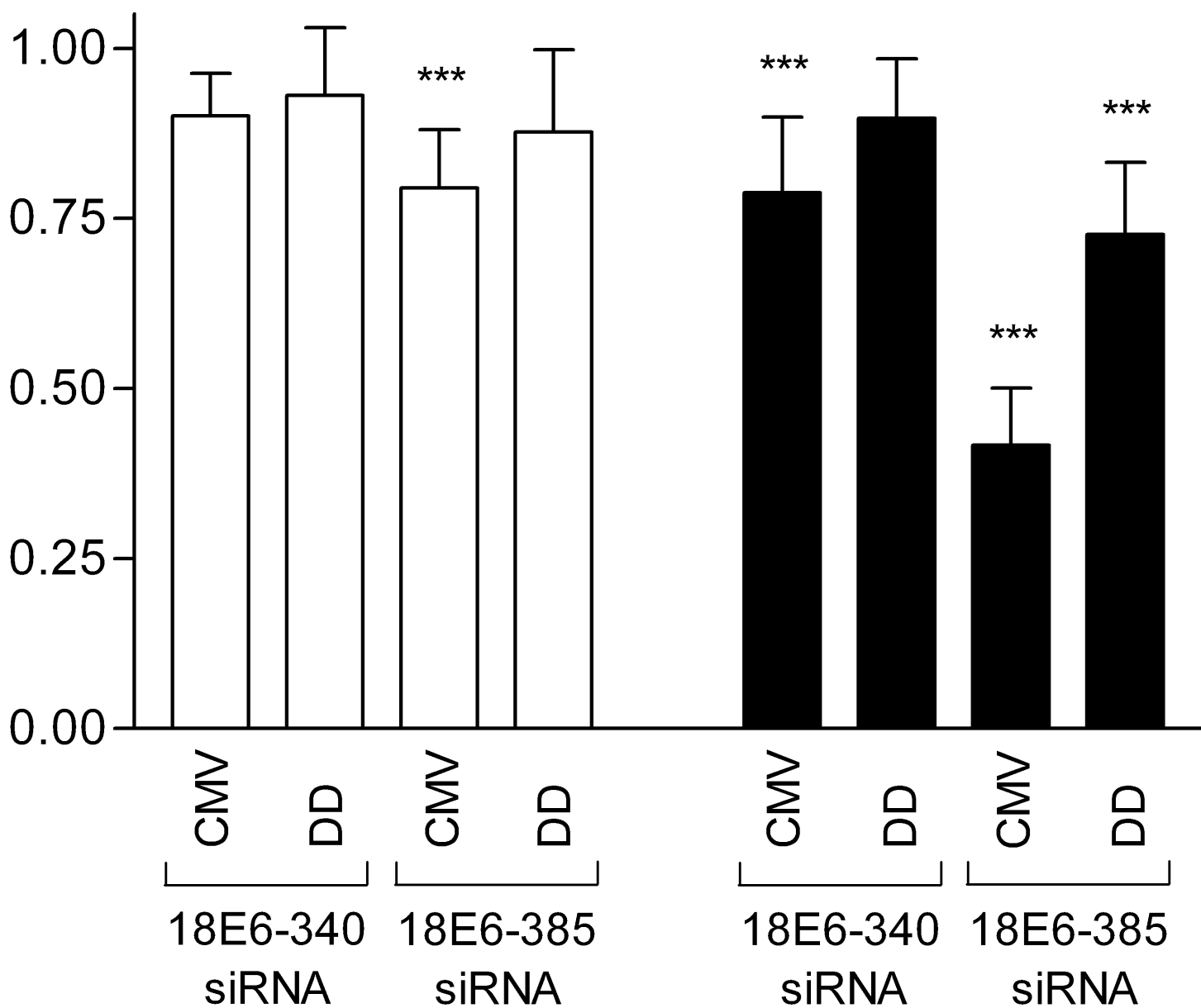
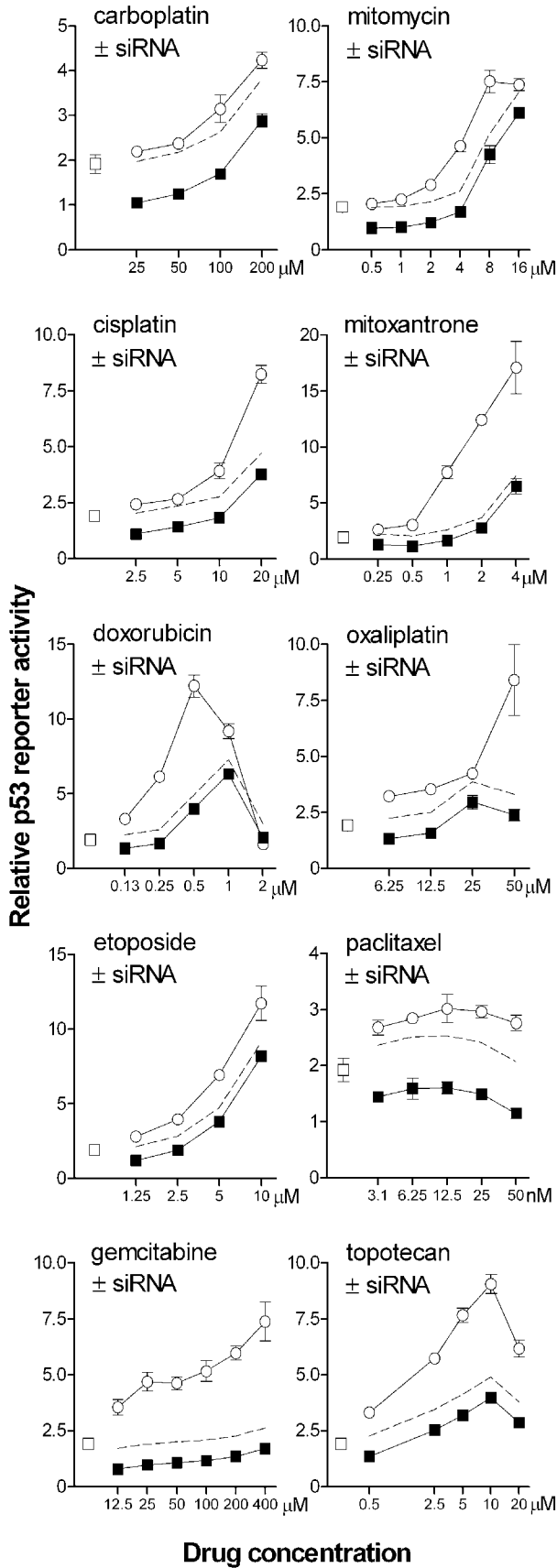


Figure 5

A



B

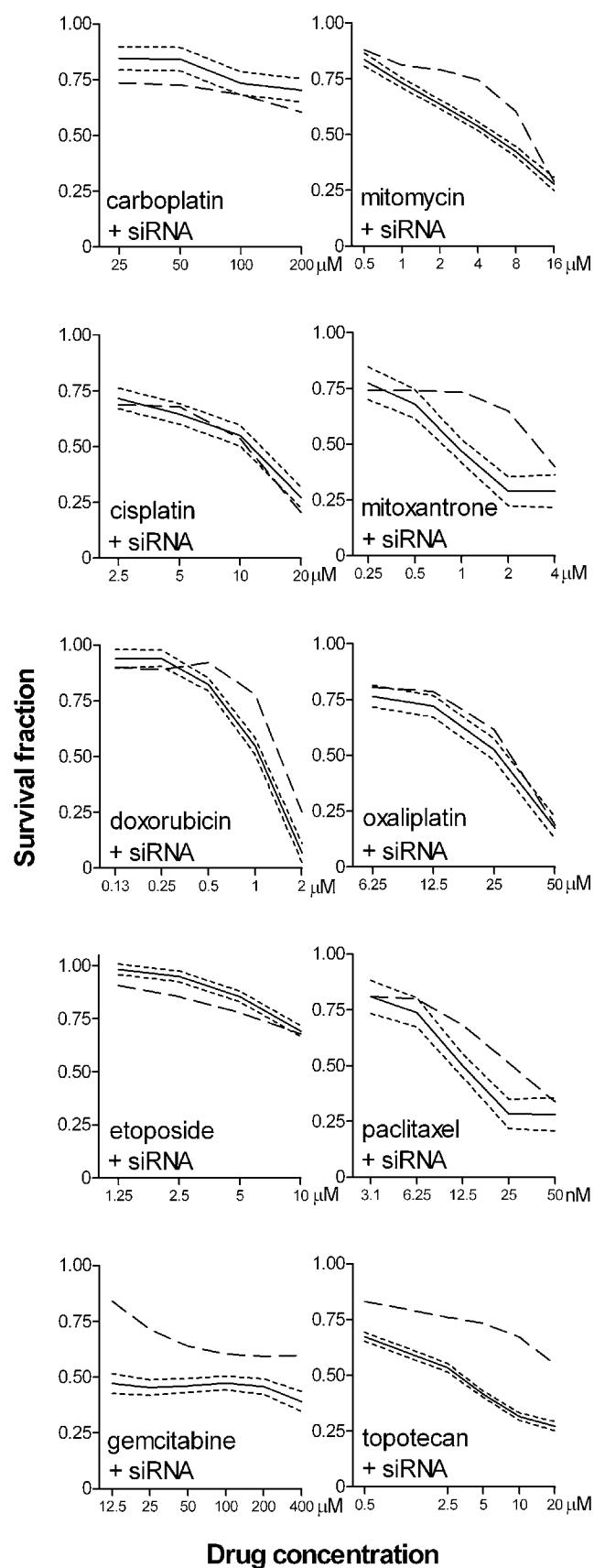


Figure 6

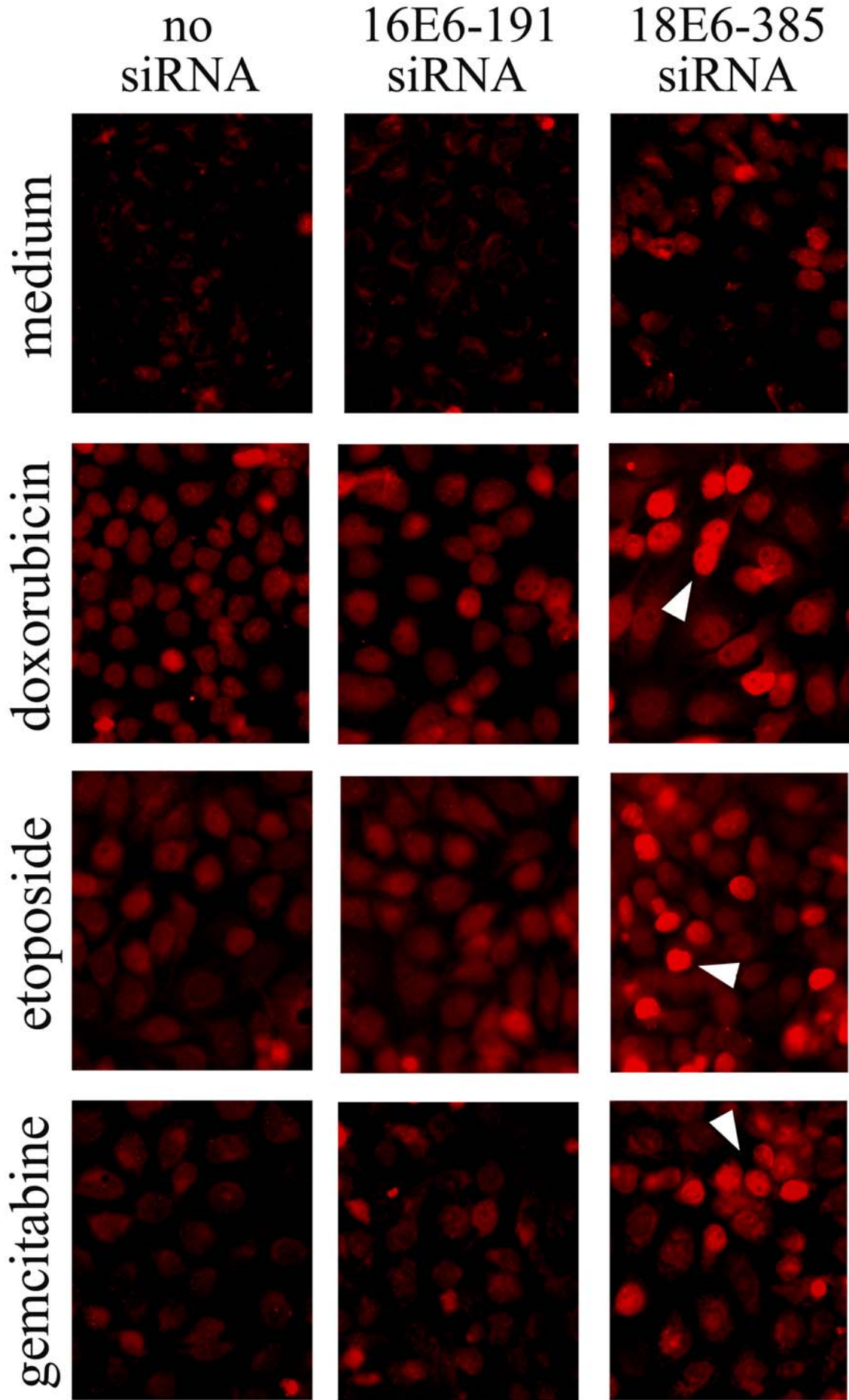
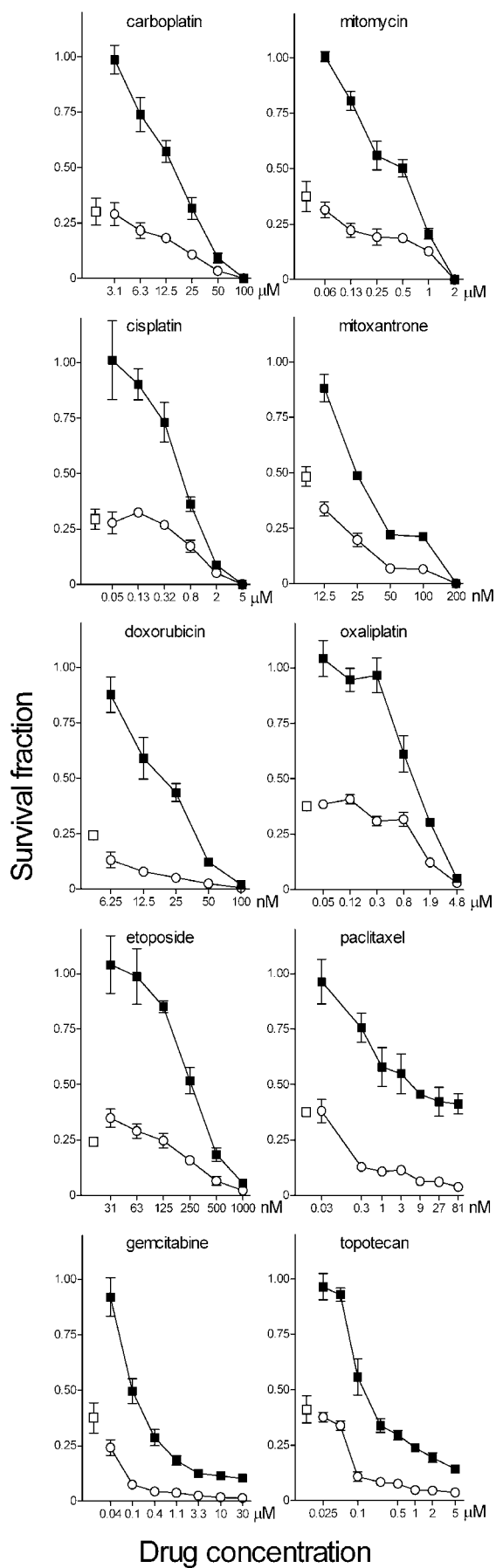


Figure 7

A



B

