Silencing of *survivin* gene by small interfering RNAs produces supra-additive growth suppression in combination with 17-allylamino-17-demethoxygeldanamycin in human prostate cancer cells

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Abstract

Survivin **is an antiapoptotic gene, which is overexpressed in most human tumors and involved in mitotic checkpoint control. Recent evidence points to an essential role for heat shock protein 90 (Hsp90) in survivin function regulation. Although the survivin-Hsp90 association may promote tumor cell proliferation, it may also suggest new opportunities for the design of novel anticancer approaches. We evaluated the effect of small interfering RNA (siRNA)–mediated inhibition of survivin on the proliferative potential of prostate cancer cells and their sensitivity to the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG). Human androgen-independent prostate cancer cell lines (DU145 and PC-3) were transfected with four 21-mer double-stranded siRNAs (100 nmol/L) directed against different portions of survivin mRNA. After transfection, cells were collected and analyzed for survivin mRNA and protein expression, cell proliferation rate, ability to undergo apoptosis, and sensitivity to 17-AAG. Transfection of prostate cancer cells with siRNAs induced a variable extent of inhibition of survivin mRNA expression (39–60% compared with controls), which was paralleled by a 38% to 75% reduction in survivin protein abundance. The three siRNAs able to induce the greatest inhibition of survivin expression also significantly reduced cell proliferation and enhanced the rate of apoptosis, with a concomitant increase in caspase-9 activity. Sequential treatment with siRNA and 17-AAG induced supra-additive antiproliferative effects in all cell lines, with an enhanced caspase-9-dependent apoptotic response. These findings suggest that combined strategies aimed at interfering with the survivin-Hsp90 connection may provide novel approaches for treatment of androgen-independent prostate cancer. [Mol Cancer Ther 2006;5(1):179–86]**

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Introduction

Survivin is a structurally unique member of the inhibitor of apoptosis protein family, which is involved in the control of mitotic progression and inhibition of apoptosis (1). Its marked expression in cancer versus normal tissues (2) and its association with unfavorable disease outcome (2) have made survivin a promising new target for anticancer interventions (3). Survivin antagonists have shown antitumor efficacy in *in vitro* and *in vivo* experimental models of several human tumor types (3) and a survivin antisense oligonucleotide recently entered clinical testing.

It has been shown recently that the association of survivin with the heat shock protein 90 (Hsp90) is required for its stability and function (4). Targeted antibody-mediated disruption of the survivin-Hsp90 complex in cancer cells resulted in proteasomal degradation of survivin, mitochondrial-dependent apoptosis, and mitotic arrest (4). Hsp90 plays a central role in the cellular stress response, which is constitutively up-regulated in cancer cells to enhance adaptation to environmental challenges (5). Specifically, Hsp90 is involved in the stabilization and conformational maturation of several proteins belonging to signal transduction pathways, which are deregulated in cancers and contribute to all major components of the malignant phenotype (6).

The identification of survivin as a new client protein for Hsp90 links the cellular stress response to the dual cell viability/mitotic checkpoint maintained by survivin. Although the survivin-Hsp90 association may promote proliferation of tumor cells by elevating their antiapoptotic threshold, it may also suggest new opportunities for the design of novel anticancer strategies. In this context, combined approaches aimed at interfering with different levels of the survivin-Hsp90 connection could be particularly useful. Here, we evaluated the effect of small interfering

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RNA (siRNA)–mediated silencing of survivin on the proliferative potential of human androgen-independent prostate cancer cells and their sensitivity to the first-in-class Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG). The results indicate that (*a*) survivin down-regulation significantly reduced cell proliferation and enhanced the rate of caspase-9-dependent apoptosis and (*b*) sequential treatment with survivin-specific siRNA followed by 17-AAG produces supra-additive antiproliferative effects in human prostate cancer cells.

Materials and Methods

Cell Lines

The *Mycoplasma*-free human prostate carcinoma cell lines DU145 and PC-3 (American Type Culture Collection, Rockville, MD) were grown as a monolayer in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 10% fetal bovine serum. The cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

siRNA Design

Four different siRNAs [siRNA2 (7), siRNA3, siRNA7, and siRNA10] targeting specific consensus sequences [5′-AA(N19)UU-3′, where N is any nucleotide] within the survivin mRNA (Genbank accession no. NM_001168.1) were designed by using the siRNA target finder tool.[1] A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) for selected siRNA sequences was done to ensure that only a single gene was targeted. A scramble siRNA, made of a sequence with no significant homology to any known human mRNA, was used as control (control siRNA) throughout the study (8). All siRNAs were purchased from Dharmacon (Ambion, Milano, Italy) as preformed and purified duplexes, made of 19 ribonucleotides with two extra thymidine bases forming a 3′ overhang on both strands (Fig. 1). Each siRNA was resuspended in water and the stock solutions (20 μmol/L) were stored at 4°C until use.

Cell Culture and Transfection

The day before transfection, prostate cancer cells were seeded at a density of 2.5×10^5 to 5.0×10^5 per 25-cm^2 flask. A given amount of each siRNA was mixed with LipofectAMINE 2000 or Oligofectamine reagent (Invitrogen, San Giuliano Milanese, Italy) for 20 minutes at room temperature according to the manufacturer's instructions. The mixtures were then applied to the cells in a final volume of Opti-MEM I (Invitrogen) giving a final concentration of each siRNA of 100 nmol/L. After incubation for 8 hours at 37°C, RPMI 1640 supplemented with serum was added. Cells were then cultured for an additional 24 to 48 hours at 37°C before further analysis.

Cell Survival Assay

To assess siRNA-mediated inhibition of cell proliferation, prostate cancer cells were transfected with 100 nmol/L of each siRNA as described above. In combination experiments, following an 8-hour transfection with siRNA2, cells were exposed for 72 hours to different concentrations (5–1,000 ng/mL) of 17-AAG (kindly provided by Dr. E. Sausville, National Cancer Institute, Bethesda, MD) previously reconstituted in sterile DMSO and then diluted with sterile water to the final dose. At the end of treatment (day 3) or after an additional 4 days in drug-free medium (day 7), cells were trypsinized and counted in a particle counter (Coulter counter, Coulter Electronics, Luton, United Kingdom). Cell viability was determined by the trypan blue dye exclusion test. The results were expressed as percent variation in the number of viable cells in treated compared with control cells.

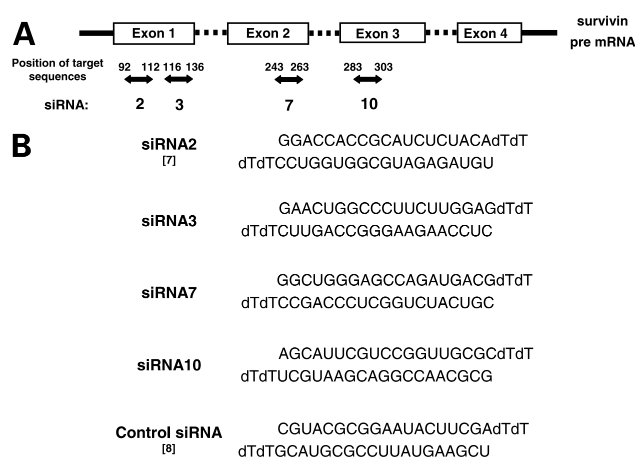

Figure 1. **A,** schematic representation of the survivin pre-mRNA and location of each target sequence in survivin mRNA (Genbank accession no. NM_001168.1). **B,** schematic structures of siRNAs. Control siRNA is a siRNA that has no significant homology to any known human mRNA (8) and was used to control the specificity of the RNA interference reaction.

RNA Preparation and Reverse Transcription-PCR

Cellular RNA was isolated from control and siRNA-transfected cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (0.25 μg) was reverse transcribed in the presence of random hexamers using the GeneAmp Reverse Transcription-PCR Core Kit (Applied Biosystems, Foster City, CA). The resultant cDNA was amplified using a specific primer pair for survivin: the sense primer was 5′-AGCCCTTTCT-CAAGGACCAC-3′ and the antisense primer was 5′-TGGCTCGTTCTCAGTGGGGCAGT-3′. Cycling conditions were as follows: initial denaturation at 95°C for 2 minutes followed by 25 cycles at 95°C for 1 minute, 62°C for 30 seconds, 72°C for 30 seconds, and 72°C for 7 minutes. A 92-bp fragment corresponding to β-actin was amplified and used as a standard for the PCR reaction. The amplified products were separated by agarose gel electrophoresis, analyzed by a ScanJET IIcx/T scanner (Hewlett Packard, Milan, Italy), and quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The relative expression level of survivin was normalized to that of β-actin.

Western Immunoblotting

Total cellular lysates were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. The

[1] http://www.ambion.com/techlib/misc/siRNA_finder

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filters were blocked in PBS with 5% skim milk and incubated overnight with the primary antibodies specific for survivin (Novus Biologicals, Littleton, United Kingdom), Akt (Cell Signaling Technology, Beverly, MA), Bcl-2, CDK6 (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-X_L, and X-linked inhibitor of apoptosis protein (Abcam Ltd., Cambridge, United Kingdom). The filters were then incubated with the secondary peroxidase-linked whole antibodies (Amersham Biosciences Europe, Freiburg, Germany). Bound antibody was detected using the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences Europe). Anti–proliferating cell nuclear antigen and anti-actin monoclonal antibodies (Santa Cruz Biotechnology) were used on each blot to ensure equal loading of protein on the gel. The results were quantified by densitometric analysis using the ImageQuant software.

Detection of Telomerase Activity

Telomerase catalytic activity was assessed on cell lysates by telomeric repeat amplification protocol assay using the TRAPeze kit (Intergen, Edinburgh, United Kingdom; ref. 9). Each reaction product was amplified in the presence of a 36-bp internal telomeric repeat amplification protocol assay standard.

Evaluation of Apoptosis

Samples of 1×10^6 cells were fixed in 70% (v/v) ethanol, stained with a solution containing 50 μg/mL propidium iodide, 10 mg/mL RNase, and 0.1% (v/v) Tween20 for 30 minutes at room temperature, and analyzed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). A flow cytometric sub-G_0-G_1 peak was detected on DNA plots using the CellQuest software according to the Modfit model (Becton Dickinson).

Caspase-9 catalytic activity was determined by the Caspase-9/Mch6 Fluorometric Protease Assay Kit (MBL Ltd., Japan). Total protein and the specific fluorogenic substrate (Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin) were mixed for 1 hour at 37°C, and substrate hydrolysis was monitored by spectrofluorometry at 505 nm.

Statistical Analysis

Student's t test was used to analyze the differences between control and siRNA-transfected cells in terms of cell proliferation, rate of apoptosis, and catalytic activity of caspase-9. All tests were two sided. Ps < 0.05 were considered statistically significant.

In combination experiments, the type of interaction between the cytotoxic effects of siRNA2 and 17-AAG was analyzed as described previously (10). For the evaluation, the agents were assumed to provide independent effects. For a given concentration of 17-AAG, we observed a survival fraction of cells (SFa); likewise, for siRNA2 transfection, we observed SFb. For the combined treatment (siRNA2 transfection followed by 17-AAG exposure), we observed SFab. The results obtained were expressed according to the following criteria: SFab = SFa × SFb indicates additive effect, SFab < SFa × SFb indicates supra-additive effect, and SFab > SFa × SFb indicates subadditive effect.

Results

Silencing of *Survivin* Gene by siRNAs Causes Cell Proliferation Decline and Apoptosis Induction in Prostate Cancer Cells

In this study, we used a RNA interference–based strategy to inhibit survivin expression in human androgen-independent prostate cancer cells. Because strong positional effects on the function of siRNAs have been shown (11), we tested the effectiveness of four 21-mer siRNAs targeting different portions within exons 1 to 3 of survivin mRNA (Fig. 1) to silence *survivin* gene expression in the DU145 androgen-independent prostate cancer cell line. Reverse transcription-PCR experiments carried out in DU145 cells collected at different intervals after a 8-hour transfection with 100 nmol/L of each survivin-specific siRNA showed a reduction of survivin mRNA compared with LipofectAMINE 2000–exposed cells (Fig. 2A). Such inhibition ranged from 39% to 60% depending on the different siRNAs at 24 hours after transfection and was still appreciable to a similar extent at 48 hours (Fig. 2C). Survivin mRNA down-regulation was paralleled by a reduction in survivin protein abundance that ranged from 38% to 75% at 24 hours and remained almost constant at 48 hours (Fig. 2B and D). Transfection of DU145 cells with 100 nmol/L of a control, unrelated siRNA caused modest inhibition (~10% compared with LipofectAMINE 2000–exposed cells) of survivin mRNA expression at both time points considered (Fig. 2C). A slightly stronger inhibitory effect (~25%) on survivin protein expression was consistently observed in cells transfected with the control siRNA (Fig. 2D). Transfection of DU145 cells with a survivin-specific siRNA, siRNA2, failed to appreciably affect the expression of other antiapoptotic proteins, including X-linked inhibitor of apoptosis protein and Bcl-X_L. As reported already (12), no Bcl-2 expression was observed in these cells (Fig. 2E).

Transfection with the three survivin-specific siRNAs (siRNA2, siRNA3, and siRNA10) able to induce the greatest inhibition of survivin expression also resulted in significant ($P < 0.01$) and time-dependent decline of DU145 cell proliferation compared with LipofectAMINE 2000–exposed cells, which was appreciable starting from 24 hours after transfection (47–55% with respect to LipofectAMINE 2000–exposed cells) and increased at 48 hours (65–73%). Conversely, transfection with the control, unrelated siRNA only induced a modest inhibitory effect on cell proliferation (data not shown).

To test whether the cytotoxic effect consequent to survivin down-regulation was due to the induction of apoptosis, we assessed by flow cytometry the presence of an apoptotic sub-G_0-G_1 peak 48 hours after transfection of DU145 with the different siRNAs. No sub-G_0-G_1 peak was observed in LipofectAMINE 2000–exposed cells, whereas a peak accounting for 6.5% of the overall cell population was present in DU145 cells transfected with the control siRNA (Fig. 3A). However, the extent of sub-G_0-G_1 peaks was markedly increased in cells transfected with the survivin-specific siRNA2, siRNA3, and siRNA10 and ranged from 25% to 40% of the overall cell population (Fig. 3A).

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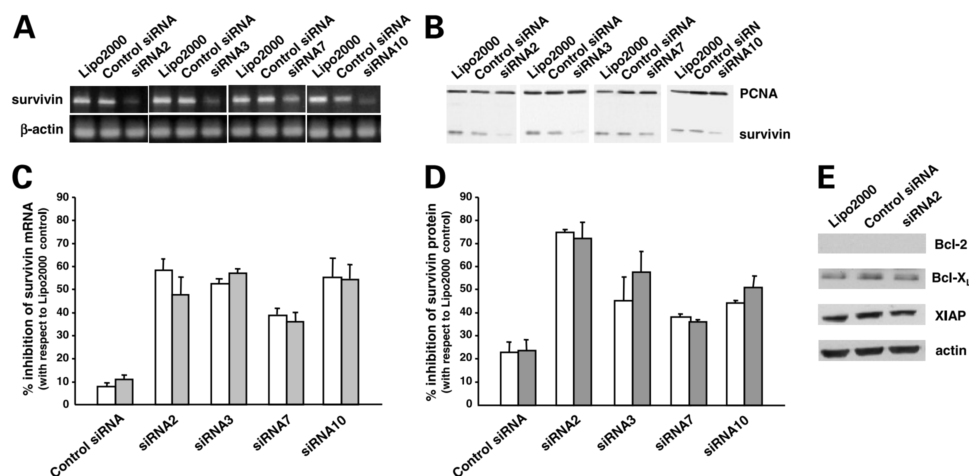

Figure 2. Representative reverse transcription-PCR (**A**) and Western blot (**B**) experiments illustrating survivin mRNA and protein expression in DU145 cells at 24 h after transfection with the different siRNAs (100 nmol/L). β-Actin and proliferating cell nuclear antigen (*PCNA*) were used as controls for loading. Quantification of survivin mRNA (**C**) and protein (**D**) expression levels at 24 h (*white columns*) and 48 h (*gray columns*) after transfection. Data are percent inhibition of survivin expression compared with LipofectAMINE 2000 – exposed cells. *Columns,* mean of three independent experiments; *bars,* SD. **E,** representative Western blot experiment illustrating the expression of other antiapoptotic proteins in DU145 cells at 24 h after transfection with 100 nmol/L siRNA2. *XIAP,* X-linked inhibitor of apoptosis.

At the molecular level, siRNA-mediated inhibition of survivin expression in DU145 cells coincided with a significantly ($P < 0.01$) increased catalytic activity of caspase-9. Specifically, the activity of the enzyme was 3.5- to 4-fold higher in DU145 cells transfected with survivin-specific siRNAs than in those exposed to LipofectAMINE 2000. Conversely, transfection of cells with the control, unrelated siRNA did not appreciably modify the extent of caspase-9 activity (Fig. 3B).

siRNA-Mediated Silencing of the *Survivin* Gene Increases the Sensitivity of Prostate Cancer Cells to 17-AAG

To test whether the basal level of survivin in the tumor cell population plays a role in determining the *in vitro* response to Hsp90 inhibitors, we examined the effect of siRNA-mediated survivin down-regulation on cell sensitivity to 17-AAG in DU145 cells. A 72-hour exposure to different drug concentrations (5–50 ng/mL) induced a

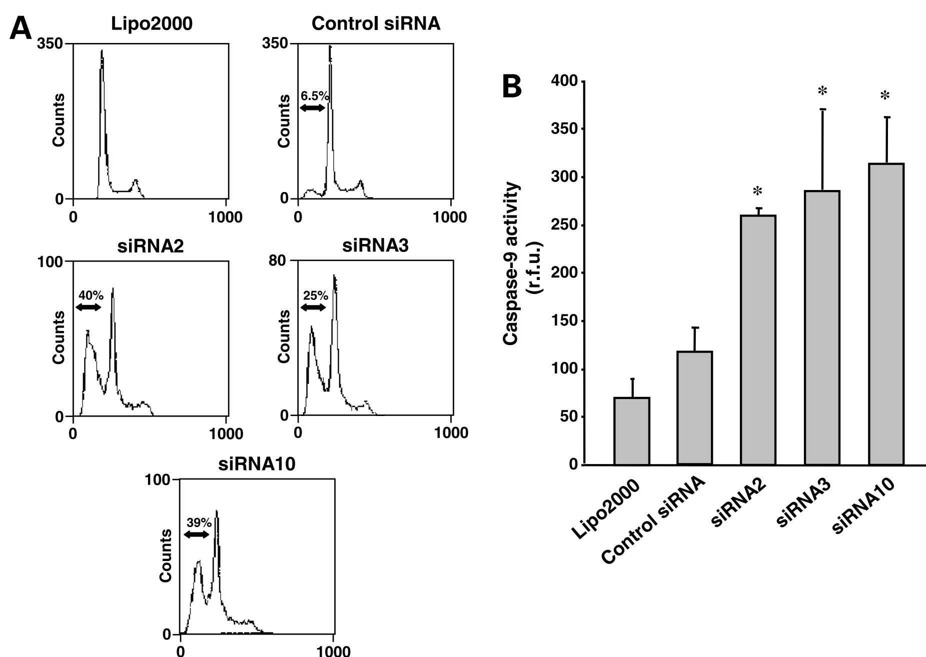

Figure 3. A, flow cytometric analysis of DU145 cells at 48 h after transfection with different siRNAs. The percentage of the pre-G_0-G_1 population is reported in each histogram. **B,** caspase-9 catalytic activity as determined by hydrolysis of the fluorogenic substrate Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin in LipofectAMINE 2000 – exposed cells and in cells transfected with different siRNAs. Data are relative fluorescence units (*r.f.u.*). *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$ (Student's *t* test).

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dose-dependent decline of DU145 cell proliferation (Fig. 4A) as well as a decrease in the expression/activity of Hsp90 client proteins, including Akt, CDK6 (Fig. 4B), and telomerase (Fig. 4C). The extent of the 17-AAG cytotoxic activity was enhanced in cells transfected with siRNA2. Specifically, at all drug concentrations tested, the sequential treatment (8-hour siRNA2 transfection followed by 72-hour 17-AAG exposure) induced an inhibitory effect on cell proliferation greater than that expected by simple additivity of the effects of the two agents, which was appreciable both at the end of treatment and at 7 days (Fig. 4A). Exposure of cells to 17-AAG alone did not appreciably affect survivin expression up to 100 ng/mL, whereas a marked and dose-dependent inhibition of protein expression was observed at the two highest drug concentrations (500 and 1,000 ng/mL; Fig. 5A and B). Sequential treatment with siRNA2 and 17-AAG consistently caused a strong decrease of survivin expression, which was almost superimposable to that observed after transfection of cells with siRNA2 alone (Fig. 5A and B). However, it was not possible to evaluate the cytotoxic effect of the sequential treatment at 17-AAG concentrations able to down-regulate survivin, because an almost complete inhibition of cell proliferation (~99%) was found after a 3-day exposure to 500 ng/mL drug.

The cytotoxic effect of 17-AAG in DU145 cells transfected with the control siRNA was superimposable to that observed in cells exposed to 17-AAG alone (data not shown).

To gain insight into the molecular basis responsible for the increased sensitivity to 17-AAG observed in siRNA2-transfected DU145 cells, we evaluated the *in vitro* catalytic activity of caspase-9 following single and combined treatments (Fig. 5C). Exposure to 17-AAG increased the activation of the enzyme compared with that observed in control (no drug) cells. However, the caspase-9 catalytic activity was consistently and significantly ($P < 0.02$) higher in cells exposed to the combined treatment (8-hour siRNA2 transfection followed by 72-hour 17-AAG exposure) than in those treated with the drug alone, and such enhancement was greatest (2.8-fold) at the highest 17-AAG concentration (Fig. 5C).

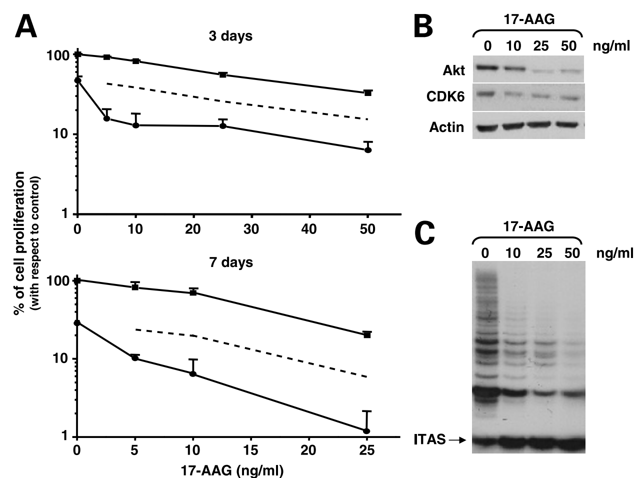

Figure 4. **A,** dose-response survival curves of DU145 cells exposed to 17-AAG alone for 72 h (■) or to combined treatment (8-h transfection with 100 nmol/L siRNA2 followed by 72-h exposure to 17-AAG; ●) and obtained at the end of treatment (3 d) or at 7 d. *Points,* mean of three independent experiments; *bars,* SD. *Dashed line,* expected curve for an additive effect of the two agents in combination. Representative Western blot (**B**) and telomeric repeat amplification protocol assay (**C**) experiments illustrating the expression of Akt and CDK6 proteins and telomerase catalytic activity in DU145 cells after a 72-h exposure to 17-AAG. *ITAS,* internal telomeric repeat amplification protocol assay standard.

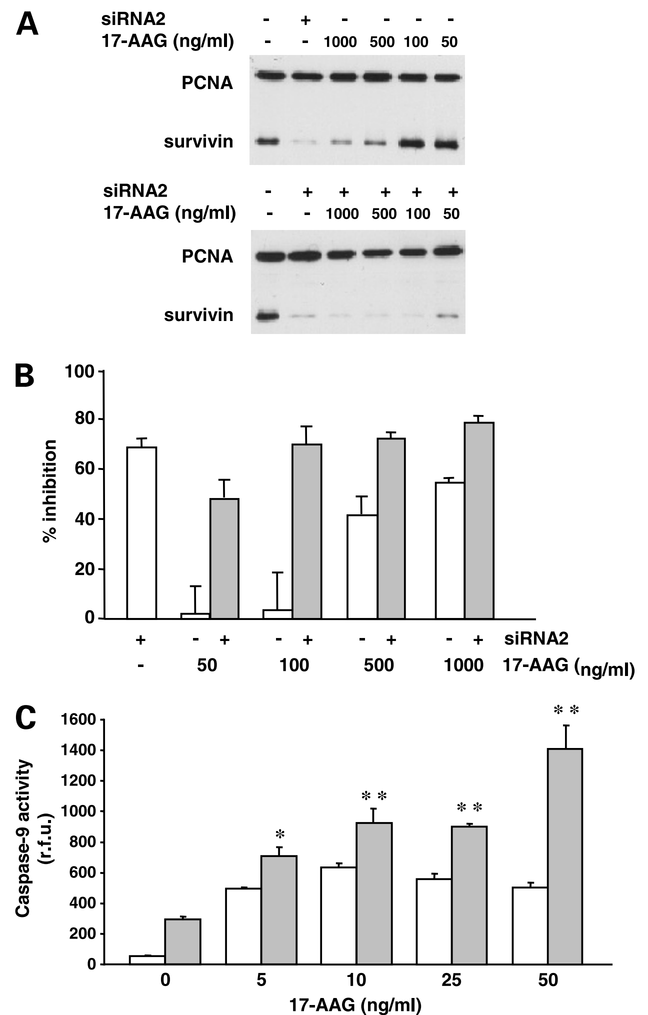

Figure 5. **A,** representative Western blot experiments illustrating the expression of survivin in DU145 cells after exposure to siRNA2 and 17-AAG singly given (*top*) or in sequence (*bottom*). **B,** quantification of survivin protein. Data are percent inhibition of survivin expression compared with LipofectAMINE 2000–exposed cells (for transfection with siRNA2 and siRNA2-17-AAG sequential treatment) or control cells (for exposure to 17-AAG alone). *Columns,* mean of three independent experiments; *bars,* SD. **C,** caspase-9 catalytic activity in DU145 cells exposed to 17-AAG alone (*empty column*) or to the combined treatment (*gray column*). *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.02$; **, $P < 0.01$ (Student's *t* test).

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To evaluate the effect of survivin down-regulation on 17-AAG sensitivity in a second prostate cancer cell line, we transfected PC-3 cells with 100 nmol/L siRNA2 for 8 hours using Oligofectamine. Such transfection induced a marked inhibition of survivin protein expression compared with Oligofectamine-exposed cells, which was appreciable starting from 24 hours after transfection and remained stable at 48 hours (Fig. 6A). Moreover, the extent of inhibition (~70–80%) was comparable with that obtained in DU145 cells after transfection with the same survivin-specific siRNA. Transfection of PC-3 cells with siRNA2 failed to appreciably modify the expression of other antiapoptotic proteins, such as X-linked inhibitor of apoptosis protein, Bcl-2, and Bcl-X_L (Fig. 6B).

PC-3 cells were considerably more resistant to 72-hour exposure to 17-AAG than DU145 cells (Fig. 6C) as indicated by the 13-fold higher IC_{50} (410 ± 20 versus 31 ± 2 ng/mL, respectively). However, 17-AAG cytotoxic activity was enhanced in cells transfected with siRNA2, and in accord with that observed in DU145 cells, the sequential treatment induced an inhibitory effect on cell proliferation (appreciable at the end of treatment and at 7 days) greater than that expected by simple additivity of the effects of the two agents when the drug was used at concentrations ranging from 100 to 500 ng/mL. Conversely, no potentiating effect was observed when the highest 17-AAG concentration (1,000 ng/mL) was used in sequence with siRNA2 (Fig. 6C). 17-AAG alone induced an appreciable inhibition of survivin expression only when used at 1,000 ng/mL, whereas a markedly decreased survivin abundance was consistently observed in cells exposed to siRNA2-17AAG sequential treatment independently of drug concentration (Fig. 6D and E).

The cytotoxic effect of 17-AAG in PC-3 cells transfected with the control siRNA was superimposable to that observed in control cells exposed to 17-AAG alone (data not shown).

Discussion

RNA interference refers to a group of related post-transcriptional gene silencing mechanisms in which the terminal effector is a short antisense RNA (13). The discovery that synthetic 21- to 23-nucleotide RNA duplexes (siRNA) can trigger an RNA interference response in mammalian cells and induce strong inhibition of specific gene expression (14) has opened the door to the therapeutic use of siRNAs. Specifically, several studies on experimental human tumor models have shown the feasibility of this approach for the inhibition of a variety of cancer-related genes (15).

Here, we showed that it is possible to markedly reduce the expression of the cytoprotective factor survivin in human androgen-independent prostate cancer cells by the

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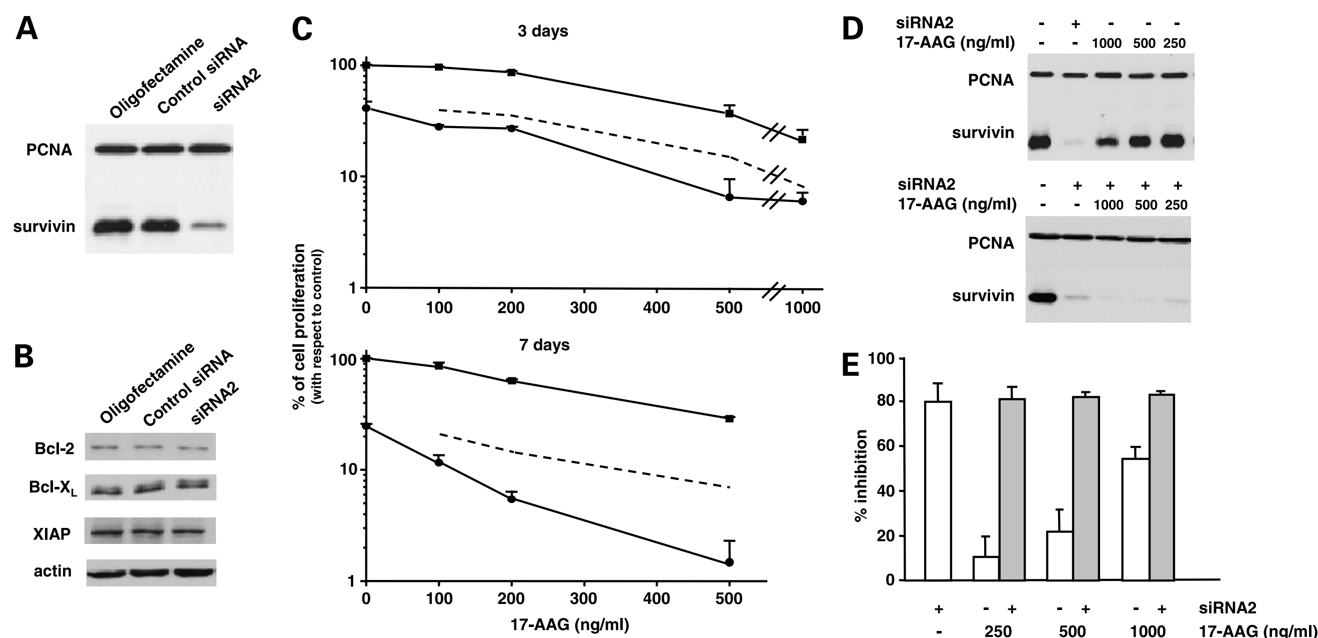

Figure 6. Representative Western blot experiments illustrating the expression of survivin (**A**) and other antiapoptotic proteins (**B**) in PC-3 cells at 24 h after transfection with siRNA2 (100 nmol/L). **C,** dose-response survival curves of PC-3 cells exposed to 17-AAG alone for 72 h (■) or to combined treatment (8-h transfection with 100 nmol/L siRNA2 followed by 72-h exposure to 17-AAG; ●) and obtained at the end of treatment (3 d) or at 7 d. *Points,* mean of three independent experiments; *bars,* SD. *Dashed line,* expected curve for an additive effect of the two agents in combination. **D,** a representative Western blot experiment illustrating the expression of survivin in PC-3 cells after exposure to siRNA2 and 17-AAG singly given (*top*) or in sequence (*bottom*). **E,** quantification of survivin protein. Data are percent inhibition of survivin expression compared with Oligofectamine-exposed cells (for transfection with siRNA2 and siRNA2-17-AAG sequential treatment) or control cells (for exposure to 17-AAG alone). *Points,* mean of three independent experiments; *bars,* SD.

use of siRNAs. Because it seems that target specificity can be attained depending on the position and sequence of a given siRNA (11), in this study, we used four 21-mer double-stranded siRNAs targeting different regions within survivin mRNA. A marked, although variable, extent of survivin inhibition in terms of mRNA and protein expression was observed with the different siRNAs, indicating the specificity of the inhibitory approach, which was also confirmed by the lack of an effect on the expression of other antiapoptotic proteins. However, the evidence of a modest but definite degree of survivin inhibition, mainly appreciable at the protein level, following transfection of cells with a control, unrelated siRNA suggests the occurrence of minor off-target effects induced by siRNAs. In this context, microarray experiments aimed at evaluating whether global gene expression patterns change in the presence or absence of siRNAs already suggested that a few nontarget transcripts may also be affected (16).

siRNA-mediated down-regulation of survivin expression in DU145 cells resulted in a significant decline of cell proliferation and an increase in the rate of spontaneous apoptosis. These findings corroborate previous evidence indicating that interference with survivin function by the use of siRNAs (17–20) and other kinds of inhibitors, including antisense oligonucleotides (21, 22), hammerhead ribozymes (23, 24), or dominant-negative mutants (25, 26), led to increased apoptotic cell death in different human tumor models. Consistent with a selective role of survivin in antagonizing mitochondrial-dependent apoptosis (27), we found a significant enhancement of *in vitro* caspase-9 catalytic activity in DU145 cells transfected with survivin-specific siRNAs.

The possibility to restore the susceptibility to programmed cell death as a consequence of *survivin* gene silencing seems particularly relevant in human androgen-independent prostate cancer cells. In fact, one of the main events associated with the conversion to an androgen-independent phenotype and the concomitant loss of susceptibility not only to hormonal manipulation but also to chemotherapy and radiotherapy is an increased resistance to apoptosis due to dysregulation of the apoptotic pathways (28). Specifically, a role for survivin in mediating resistance of prostate cancer cells to antiandrogen therapy with flutamide has been reported recently (29).

Several *in vitro* and *in vivo* studies indicated that survivin down-regulation was able to sensitize human tumor cells of different histologic origin to conventional chemotherapeutic drugs, including Taxol, doxorubicin, etoposide, and cisplatin (22–24, 30, 31), as well as to ionizing radiation (32). However, thus far, no data are available concerning the effect of survivin down-regulation on the activity of ansamycin antibiotics, such as geldanamycin and 17-AAG, which inhibit the chaperone function of Hsp90 by targeting its ATPase activity (33). Hsp90 inhibition results in proteasomal degradation of client proteins leading to potent antitumor activity in experimental models (34, 35). In fact, although Hsp90 is highly expressed in most cells, Hsp90 inhibitors selectively kill cancer cells compared with normal cells. The molecular mechanism of such tumor selectivity is the result of an activated, high-affinity conformation of Hsp90 in tumor cells (36). Hsp90 inhibitors have been used as probes to define the biological function of the chaperone at the molecular level and to validate it as a novel target for cancer therapy. One of these inhibitors, 17-AAG, has entered clinical trials, which served as proof-of-principle that Hsp90 function can be modulated pharmacologically without undue toxicity in humans (37). The search for second-generation analogues with potential preferential pharmacologic features is actively pursued. However, the best way to use Hsp90 inhibitors as anticancer agents remains to be defined also because of the limited knowledge of the determinants of cellular sensitivity to these compounds.

In the present study, we showed that the level of survivin expression influences the *in vitro* response of DU145 human prostate cancer cells to 17-AAG as suggested by the supra-additive cytotoxic effect observed after sequential treatment with a survivin-specific siRNA and the Hsp90 inhibitor, which was accompanied by a significantly increased caspase-9-dependent apoptotic response. This chemosensitizing effect seems to be independent of the inherent susceptibility to 17-AAG of the tumor cell system, because it was also observed in PC-3 cells, which are considerably more resistant to the Hsp90 inhibitor than DU145 cells. Moreover, because 17-AAG itself induces an inhibition in survivin expression only when used at high concentrations (500 and 1,000 ng/mL), it is unlikely that the supra-additive cytotoxic effects we observed with the siRNA2-17-AAG sequential treatment at lower drug concentrations were due to a cooperative effect of the two agents in inducing survivin depletion.

Our findings indicate that 17-AAG is more effective in cancer cells expressing reduced levels of survivin and suggest the opportunity to design combined treatments, including survivin inhibitors and 17-AAG, and test their antitumor potential against androgen-independent prostate cancer.

It has been reported previously that 17-AAG treatment reduced the expression of the androgen receptor in human prostate cancer xenografts and inhibited their growth in the animal (38). Specifically, 17-AAG induced degradation of both wild-type and mutant androgen receptor, thus suggesting that this drug may be particularly effective in the treatment of androgen-independent prostate cancer. In fact, in a subset of patients treated with androgen receptor agonists, clinical progression is associated with androgen receptor gene amplification or mutation (39), which may result in sufficient androgen receptor pathway activation to allow tumor growth at low levels of testosterone or following constitutive ligand-independent activation of the receptor.

The specific inhibition of survivin levels combined with the collapse of Hsp90 function by ansamycin antibiotics would be expected to exert a strong antitumor effect by

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disabling multiple signaling networks required for prostate cancer cell maintenance. However, before using RNA interference technology in human clinical trials, much work remains to be done to guarantee the specificity of siRNAs and to optimize safe and efficacious systems for their delivery. In this context, the possibility to convert survivin inhibitors into drug like small molecules could open important opportunities for their clinical use.

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