

Ribozyme-mediated inhibition of survivin expression increases spontaneous and drug-induced apoptosis and decreases the tumorigenic potential of human prostate cancer cells

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Survivin is a member of the inhibitor of apoptosis protein (IAP) family, which has been implicated in inhibition of apoptosis and control of mitotic progression. The finding that survivin is overexpressed in most human tumors but absent in normal adult tissues has led to the proposal of survivin as a promising therapeutic target for anticancer therapies. We decided to evaluate the effects of a ribozyme-based strategy for survivin inhibition in androgen-independent human prostate cancer cells. We constructed a Moloney-based retroviral vector expressing a ribozyme targeting the 3' end of the CUA₁₁₀ triplet in survivin mRNA, encoded as a chimeric RNA within adenoviral VA1 RNA. Polyclonal cell populations obtained by infection with the retroviral vector of two androgen-independent human prostate cancer cell lines (DU145 and PC-3) were selected for the study. Ribozyme-expressing prostate cancer cells were characterized by a significant reduction of survivin expression compared to parental cells transduced with a control ribozyme; the cells became polyploid, underwent caspase-9-dependent apoptosis and showed an altered pattern of gene expression, as detected by oligonucleotide array analysis. Survivin inhibition also increased the susceptibility of prostate cancer cells to cisplatin-induced apoptosis and prevented tumor formation when cells were xenografted in athymic nude mice. These findings suggest that manipulation of the antiapoptotic survivin pathway may provide a novel approach for the treatment of androgen-independent prostate cancer.

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Introduction

Prostate cancer is the most frequently diagnosed tumor and the second leading cause of cancer-related death among men in Western countries (Dennis and Resnick, 2000). Whereas more than 80% of tumors are initially responsive to androgen ablation, metastatic disease inevitably progresses to an androgen-independent state that is largely refractory not only to hormonal manipulation but also to chemotherapy and radiotherapy (Oh and Kantoff, 1998). Accordingly, androgen-independent progression is the main obstacle to survival in patients with advanced disease, and this emphasizes the need for novel therapeutic strategies targeting the molecular determinants of treatment resistance of advanced prostate cancer. Several lines of evidence suggested that one of the main events associated with the conversion to an androgen-independent phenotype is increased resistance to apoptosis (Denmeade *et al.*, 1996, Howell, 2000), mainly due to upregulation of antiapoptotic genes, including *Bcl-2*, *Bcl-X_L* and *Mcl-1* (Krajewska *et al.*, 1996). As a consequence, the identification of points in the apoptotic pathway at which dysregulation occurs could open new therapeutic opportunities for this malignancy.

Survivin is a structurally unique member of the inhibitor of apoptosis protein (IAP) family (Ambrosini *et al.*, 1997), which is involved in control of cell division and inhibition of apoptosis (Altieri and Marchisio, 1999). This protein is strongly expressed in embryonic and fetal organs, but undetectable in most terminally differentiated normal tissues (Altieri, 2001). Moreover, it has been described to be selectively expressed in the most common human neoplasms, including prostate cancer (McEleny *et al.*, 2002), and to be associated with clinical tumor progression (Altieri, 2001). As regards the precise role of survivin in programmed cell death, at present it is still unclear whether the protein inhibits caspases through direct binding, as other IAPs do, or indirectly, requiring intermediate proteins (Song *et al.*, 2003). Survivin shows a clear cell cycle-dependent expression, which is controlled at transcriptional (Li and Altieri, 1999) and post-translational (O'Connor

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et al., 2000) levels. When expressed at mitosis, survivin localizes to various components of the mitotic apparatus and physically associates with microtubules of the mitotic spindle (Giodini *et al.*, 2002). Accumulating evidence supports the existence of a multifunctional survivin pathway positioned at the interface between mitotic progression and apoptosis inhibition and required to preserve the viability of proliferating tumor cells (Altieri, 2003a). Survivin also appears to be involved in tumor cell resistance to some anticancer agents as well as ionizing radiation (Asanuma *et al.*, 2000; Zaffaroni *et al.*, 2002). On the basis of these findings, survivin has been proposed as a promising target for new anticancer interventions (Altieri, 2003b). In *in vitro* and *in vivo* studies targeting survivin with antisense oligonucleotides (Chen *et al.*, 2000; Olie *et al.*, 2000) or dominant-negative mutants (Grossman *et al.*, 2001; Mesri *et al.*, 2001) reduced tumor-growth potential and sensitized tumor cells to chemotherapeutic drugs such as taxol, cisplatin and etoposide.

As an alternative strategy to target the survivin pathway, we developed a Moloney-based retroviral vector expressing a hammerhead ribozyme directed against the 3' end of the CUA₁₁₀ triplet in survivin mRNA, encoded as a chimeric RNA within adenoviral VA1 RNA. Here, we demonstrate that ribozyme-mediated inhibition of survivin expression in androgen-independent human prostate cancer cells resulted in caspase-9-dependent apoptosis and suppressed tumor growth when cells were xenografted in athymic mice.

Results

Construction of a retroviral vector for targeted intracellular expression of an antisurvivin ribozyme and transduction of prostate cancer cells

A hammerhead ribozyme directed against the 3' end of the CUA₁₁₀ triplet located in exon 1 of survivin mRNA was used in the study (Figure 1a). *In vitro* analysis of substrate specificity and functional activity of this ribozyme has been previously reported (Pennati *et al.*, 2003). To obtain intracellular expression, the ribozyme coding sequence was cloned as a substitute to a portion of the central domain of the adenovirus type 2 VA1 gene (Figure 1b), a region that does not influence transcription by RNA polymerase III or cellular localization of the RNA in the cytoplasm (Cagnon and Rossi, 2000; Mendoza-Maldonado *et al.*, 2002). Moreover, the catalytic activity of the ribozyme was retained, as assessed in *in vitro* cleavage assay (data not shown). As a control, we also inserted in the VA1 construct an irrelevant ribozyme targeting the feline immunodeficiency virus (FIV) primer binding site. To attain high-efficiency expression of the VA1-ribozyme cassettes, Moloney-based retroviral vectors were constructed, in which the VA1-ribozyme cassette was encoded in the 3' long terminal repeat (LTR). After transduction, a double copy vector was obtained, with the ribozyme-expressing cassettes at both LTRs.

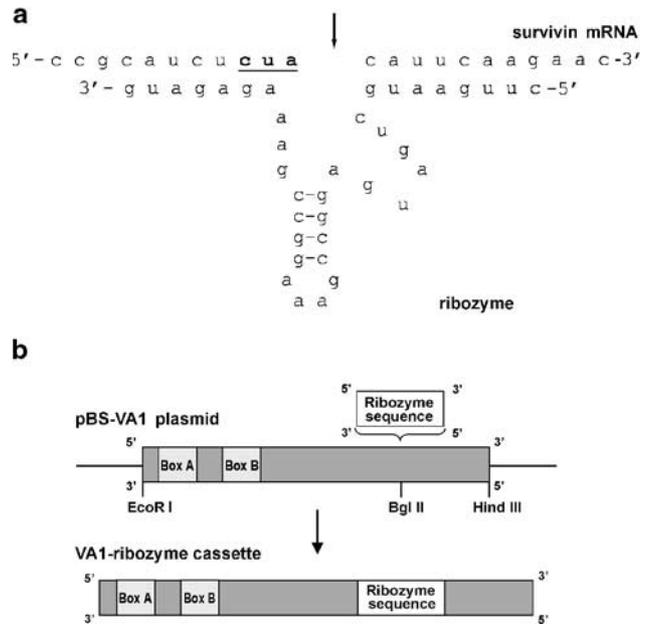


Figure 1 (a) Schematic representation of base pairing of the ribozyme with its RNA target sequence corresponding to a portion of the survivin mRNA. The cleavage site is indicated by an arrow. (b) The VA1-ribozyme cassette was obtained by inserting the ribozyme-coding sequence at the *Bgl*II site of the adenovirus type 2 VA1 gene, previously cloned in the *pBS* plasmid. Box A and Box B represent the RNA pol III promoter

Two androgen-independent human prostate cancer cell lines, DU145 and PC-3, were transduced with the viral vectors and treated *in vitro* with G418 for 1 month. G418-resistant clones were selected and screened for survivin expression by Western blotting. To rule out the possibility that attenuation of survivin expression was simply due to clonal divergence, we used polyclonal populations proven to endogenously express the anti-survivin (DU145/RZ and PC-3/RZ cells) or control (DU145/CTR and PC-3/CTR cells) ribozyme (Figure 2a). Specifically, DU145/RZ and PC-3/RZ cells were characterized by a markedly lower survivin protein level (-90 ± 8 and $-68 \pm 5\%$, respectively) than DU145/CTR and PC-3/CTR cells (Figure 2b), as assessed in three independent Western blot experiments. Attenuation of survivin protein expression markedly affected the *in vitro* growth potential of DU145/RZ cells, as demonstrated by the significantly ($P < 0.01$) longer doubling time than DU145/CTR cells (48 ± 4 vs 26 ± 3 h). Consistent with the role of survivin in the proper execution of mitosis (Giodini *et al.*, 2002), we observed that the almost complete ribozyme-mediated inhibition of survivin induced aberrant mitotic progression in DU145/RZ cells with the appearance of a fraction of polyploid cells characterized by a more than 4N DNA content (Figure 3). This finding was consistently observed in four cell samples collected at different intervals of growth in culture. Conversely, a very modest increase in the doubling time of PC-3/RZ compared to PC-3/CTR cells (30 ± 3 vs 24 ± 3 h) was observed, and the presence of polyploid cells was

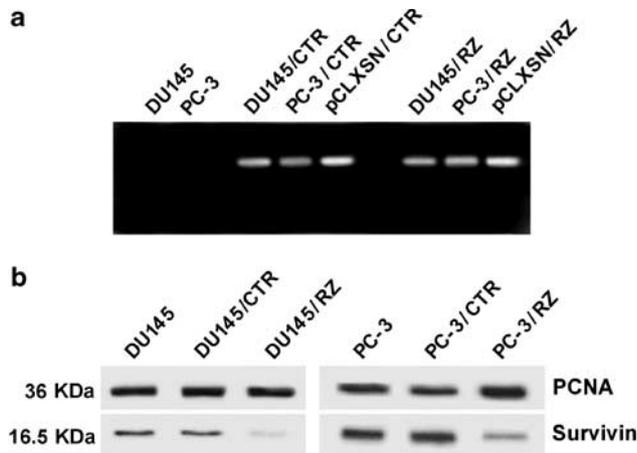


Figure 2 Ribozyme and survivin expression in prostate cancer cells. (a) Ribozyme expression was detected by RT-PCR in DU145 and PC-3 parental cells and cell clones transduced with the antisurvivin ribozyme (DU145/RZ and PC-3/RZ) or with the control ribozyme (DU145/CTR and PC-3/CTR). *pCLXSN/CTR* and *pCLXSN/RZ* plasmids were used as controls for the correct size of each fragment during PCR amplification. (b) A representative Western blotting experiment illustrating the expression of survivin in DU145, DU145/CTR, DU145/RZ, PC-3, PC-3/CTR and PC-3/RZ cells. PCNA was used as a control for loading

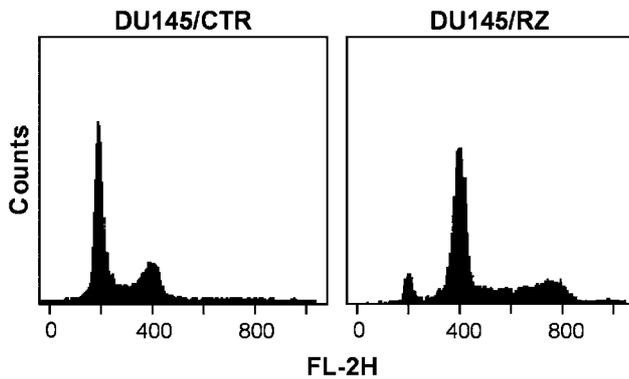


Figure 3 Effect of survivin inhibition on cell cycle progression. Cell cycle analysis of DU145/CTR (left panel) and DU145/RZ (right panel) was carried out by flow cytometry on cells stained with propidium iodide

undetectable in the PC-3/RZ cell population (data not shown).

Ribozyme-mediated survivin inhibition enhances spontaneous and drug-induced apoptosis in prostate cancer cells

Downregulation of survivin in ribozyme-expressing prostate cancer cells was associated with an increased rate of spontaneous apoptosis in both cell lines. Specifically, a more marked DNA fragmentation was observed in cells expressing the antisurvivin ribozyme, as indicated by the percentage of Tdt-mediated dUTP nick-end labeling (TUNEL)-positive cells, which was 20% in DU145/RZ and 17% in PC-3/RZ compared to only 2 and 3% in DU145/CTR and PC-3/CTR cells, respectively (Figure 4a). At the molecular level, ribo-

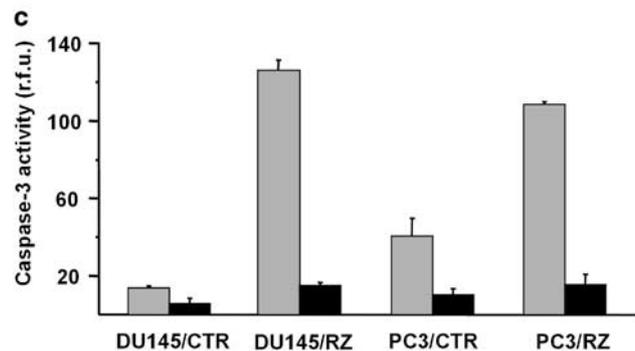
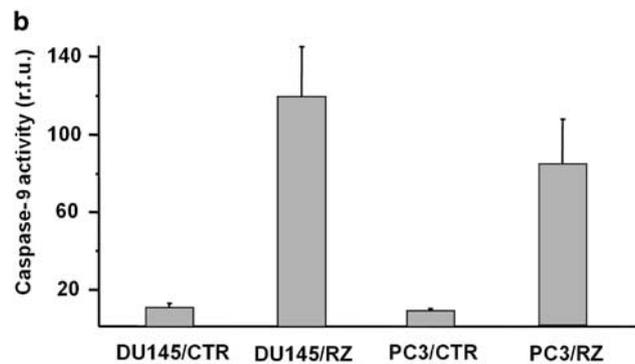
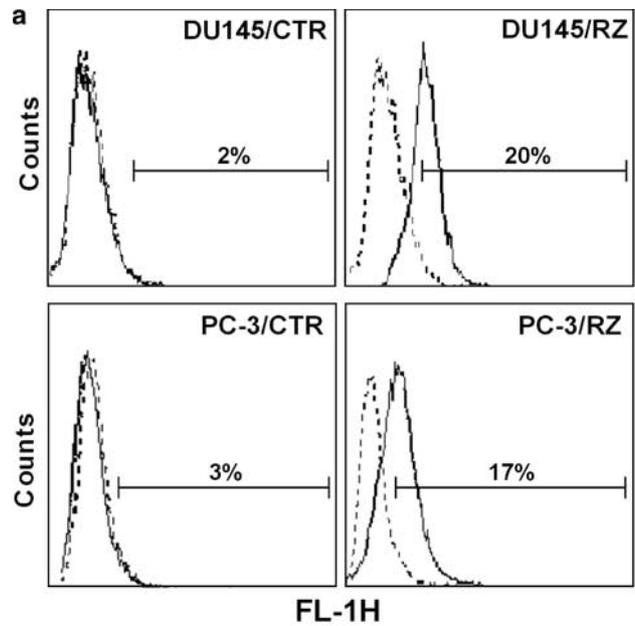


Figure 4 Ribozyme-mediated inhibition of survivin expression induces apoptosis. (a) TUNEL analysis of spontaneous apoptosis in DU145/CTR, DU145/RZ, PC-3/CTR and PC-3/RZ cells was carried out by flow cytometry. Broken lines represent the negative control incubated in the absence of terminal transferase; solid lines represent the samples incubated with TUNEL reaction mixture. The percentage of TUNEL-positive cells in each sample is reported. (b) Caspase-9 catalytic activity was determined by hydrolysis of the fluorogenic substrate LEHD-AFC. The data are expressed as relative fluorescence units (r.f.u.) and represent mean values \pm s.d. of three independent experiments. (c) Caspase-3 catalytic activity was determined by hydrolysis of the fluorogenic substrate Ac-DEVD-AMC in the absence (gray column) or presence (black column) of the caspase inhibitor (Ac-DEVD-CHO). The data represent mean values \pm s.d. of three independent experiments

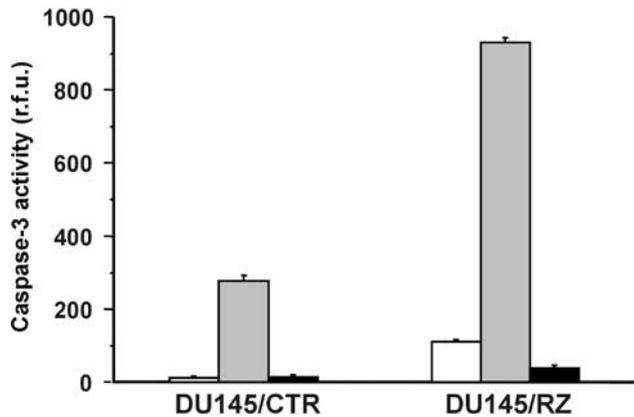


Figure 5 Ribozyme-mediated inhibition of survivin expression sensitizes prostate cancer cells to cisplatin-induced apoptosis. Caspase-3 catalytic activity was determined by hydrolysis of the fluorogenic substrate Ac-DEVD-AMC in untreated cells (empty column) and in cisplatin-treated cells in the absence (gray column) or presence (black column) of the caspase inhibitor (Ac-DEVD-CHO). The data represent mean values \pm s.d. of three independent experiments

zyme-mediated inhibition of survivin expression in DU145/RZ cells resulted in proteolytic processing of caspase-9 and caspase-3 (data not shown), which coincided with a significantly increased catalytic activity of both enzymes. Specifically, caspase-9 and caspase-3 activity, as assessed by *in vitro* hydrolysis of specific fluorogenic substrates (LEHD-AFC for caspase-9 and Ac-DEVD-AMC for caspase-3), was 12-fold ($P < 0.05$) and 10-fold ($P < 0.01$) higher, respectively, in DU145/RZ than in DU145/CTR cells (Figure 4b,c). Although to a lower extent, increased caspase-9 and caspase-3 catalytic activity (10-fold ($P < 0.01$) and threefold ($P < 0.02$), respectively) was also observed in PC-3/RZ compared to PC-3/CTR cells (Figure 4b,c).

To evaluate whether or not inhibition of survivin expression influences the susceptibility of prostate cancer cells to undergo programmed cell death after exposure to anticancer agents, we treated DU145/RZ and DU145/CTR cells with an equimolar dose (10 μ g/ml) of cisplatin and assessed the effect of drug treatment on caspase-3 activity 72 h later. Cisplatin exposure enhanced caspase-3 activity in both cell clones; however, in DU145/RZ cells expressing the antisurvivin ribozyme, the enzyme's catalytic activity was significantly ($P < 0.01$) higher than that in DU145/CTR cells (Figure 5).

Alterations in gene expression profile of prostate cancer cells expressing the antisurvivin ribozyme

The Affymetrix human genome U133A Chips were used to profile the changes in gene expression following ribozyme-mediated inhibition of survivin expression in prostate cancer cells. Pairwise comparative analysis between DU145/RZ and DU145/CTR cells was performed with the Affymetrix Microarray Suite software, which determines whether a given gene is differentially expressed based on a decision matrix including the net

change in intensity values, fold of change and other parameters. Genes with expression changes of ≥ 2.0 or ≤ 0.5 were considered as genes susceptible to modulation following specific interference with survivin expression levels. A total of 101 genes belonging to different functional categories (apoptosis, cell cycle, DNA replication and synthesis, chromosome and mitosis, transcription, signal transduction and cytoskeleton) were identified as differentially expressed (Table 1). In

Table 1 List of modulated genes following ribozyme-mediated inhibition of survivin expression

Affymetrix probe set	Gene/protein
<i>Repressed genes</i>	
202095_s_at	Survivin ^a
206504_at	Cytochrome P450, subfamily XXIV
203438_at	Stanniocalcin 2
210095_s_at	Insulin-like growth factor binding protein 3
201839_s_at	Tumor-associated calcium signal transducer 1
210654_at	Tumor necrosis factor receptor superfamily, member 10d
200606_at	Desmoplakin (DPI, DPII)
41660_at	Cadherin, EGF LAG seven-pass G-type receptor 1
217892_s_at	Epithelial protein lost in neoplasm β -actin-cytoskeleton associated
210538_s_at	Baculoviral IAP repeat-containing 3 (MIHC)
204115_at	Guanine nucleotide binding protein 11
201170_s_at	Basic helix-loop-helix domain containing, class B, 2 (BHLHB2)
203373_at	STAT-induced STAT inhibitor-2
211804_s_at	Cyclin-dependent kinase 2 (CDK2) ^a
202246_s_at	Cyclin-dependent kinase 4 (CDK4)
202705_at	Cyclin B2
204237_at	CED-6 protein
214710_s_at	Cyclin B1 ^a
217979_at	Tetraspan NET-6 protein
201565_s_at	Inhibitor of DNA binding 2
218854_at	Squamous cell carcinoma antigen recognized by T cell
210372_s_at	Tumor protein D52-like 1
203444_s_at	Metastasis-associated 1-like 1
211559_s_at	Cyclin G2
202388_at	Regulator of G-protein signalling 2
207042_at	E2F family transcription factor 2
202667_s_at	HLA class II region expressed gene KE4
204947_at	E2F family transcription factor 1 ^a
201853_s_at	Cell division cycle 25B (cdc25B) ^a
205321_at	Eukaryotic translation initiation factor 2
219032_x_at	Opsin 3 (encephalopsin, panopsin)
203209_at	Replication factor C (activator 1) 5
202437_s_at	Cytochrome P450, subfamily I
203176_s_at	Transcription factor A, mitochondrial
201659_s_at	ADP-ribosylation factor-like 1
201645_at	Tenascin C
209904_at	Troponin C
212139_at	GCN1
201131_s_at	Cadherin 1, type 1, E-cadherin
203832_at	Small nuclear ribonucleoprotein polypeptide F
203665_at	Heme oxygenase (decycling) 1
200734_s_at	ADP-ribosylation factor 3
201055_s_at	Heterogeneous nuclear ribonucleoprotein A0
214023_x_at	Tubulin, beta (β -tubulin) ^a
201475_x_at	Methionine-tRNA synthetase
202076_at	Baculoviral IAP repeat-containing 2 (BIRC2)
205016_at	Transforming growth factor, alpha (TGFA)
202241_at	Phosphoprotein regulated by mitogenic pathways
202857_at	Transmembrane protein 4
203276_at	Lamin B1
206924_at	Interleukin 11

Table 1 Continued

Affimetrix probe set	Gene/protein
200779_at	Activating transcription factor 4
202554_s_at	Glutathione S-transferase M3
203851_at	Insulin-like growth factor binding protein 6
203814_s_at	NAD(P)H dehydrogenase, quinone 2
208296_x_at	TNF-induced protein antiapoptosis (GG2-1)
206581_at	Basonuclin
206205_at	M phase phosphoprotein 9
201524_x_at	Ubiquitin-conjugating enzyme E2N
201508_at	Insulin-like growth factor binding protein 4
203752_s_at	jun D
202569_s_at	MAP/microtubule affinity-regulating kinase 3
201848_s_at	BCL2/adenovirus E1B 19kDa interacting protein (BNIP3)
200046_at	Defender against cell death 1 (DAD1)
204696_s_at	Cell division cycle 25A (cdc25A)
203628_at	Insulin-like growth factor 1 receptor
201938_at	CDK2-associated protein 1
207766_at	Cyclin-dependent kinase-like 1 (CDC2-related kinase)
200707_at	Protein kinase C substrate 80K-H
213209_at	TAF6-like RNA polymerase II
200750_s_at	RAN, member RAS oncogene family
221566_s_at	Nucleolar protein 3 (apoptosis repressor with CARD domain)
203928_x_at	Microtubule-associated protein tau
213829_x_at	Tumor necrosis factor receptor superfamily, member 6b
<i>Induced genes</i>	
204298_s_at	Lysyl oxidase
204493_at	BH3 interacting domain death agains (BID) ^a
204363_at	Coagulation factor III
201842_s_at	EGF-containing fibulin-like extracellular matrix protein 1
201324_at	Epithelial membrane protein 1
202177_at	Growth arrest-specific 6 (GAS6)
201058_s_at	Myosin
205347_s_at	Thymosin, β
202644_s_at	Tumor necrosis factor, α -induced protein 3
203725_at	Growth arrest and DNA-damage-inducible, α
203743_s_at	Thymine-DNA glycosylase
203060_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2
206116_s_at	Tropomyosin 1 (α)
209834_at	carbohydrate (chondroitin 6) sulfotransferase 3
202695_s_at	Serine/threonine kinase 17a
209008_x_at	Keratin 8
212481_s_at	Tropomyosin 4
207071_s_at	Aconitase 1
202668_at	Ephrin-B2
210762_s_at	Deleted in liver cancer 1
209369_at	Annexin A3
201954_at	Actin related protein 2/3 complex, subunit 1B
201379_s_at	Tumor protein D52-like 2
200982_s_at	Annexin A6
201980_s_at	Ras suppressor protein 1
202217_at	ES1 (zebrafish) protein, human homolog
201301_s_at	Annexin A4

An expression change of ≤ 0.5 or ≥ 2.0 was chosen as the criterion to define gene repression or induction.

^aConfirmed by Western blotting

all, 74 (73%) of them were repressed and 27 (27%) were induced following survivin inhibition. Western blot analysis, carried out on three independent replicates, was used to confirm the changes in expression of a subset of nine genes dealing with cell cycle (CDK2, CDK4, cyclin B1, cdc25A, cdc25B and GAS6), transcrip-

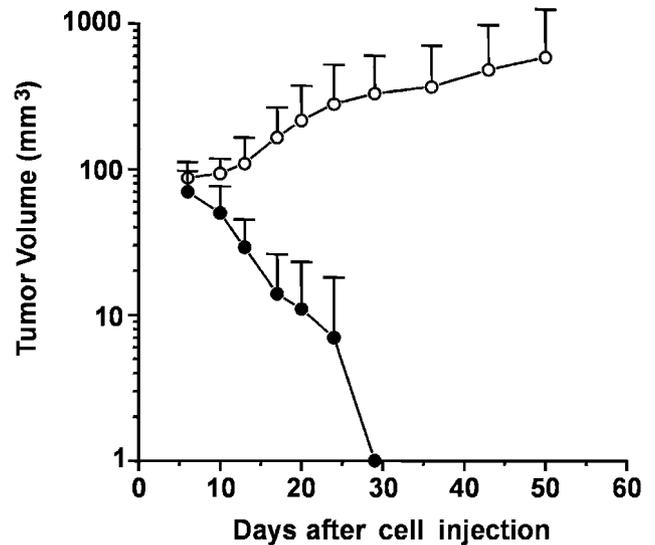


Figure 6 Effect of ribozyme-mediated survivin inhibition on the tumorigenic potential of human prostate cancer cells. Exponentially growing DU145/CTR (○) and DU145/RZ (●) cells were injected subcutaneously (10^7 cells/mouse) into the right flank of male athymic nude mice. Tumor volumes were calculated as described in 'Materials and methods'

tion (E2F-1), apoptosis (BID) and cytoskeleton (β -tubulin). The protein expression changes of six of the nine genes correlated well with the array data, indicating decreased expression of CDK2 ($-40 \pm 8\%$), cyclin B1 ($-75 \pm 10\%$), cdc25B ($-30 \pm 5\%$), E2F-1 ($-82 \pm 15\%$) and β -tubulin ($-30 \pm 9\%$), and increased expression of BID ($+88 \pm 8\%$) in DU145/RZ compared to DU145/CTR cells (data not shown).

Effect of survivin inhibition on tumorigenic potential of prostate cancer cells

The effect of ribozyme-mediated inhibition of survivin expression on tumor growth *in vivo* was studied in a xenograft prostate cancer model. Subcutaneous injection of DU145/CTR cells into athymic nude mice induced tumor growth in nine of nine xenografted mice. Tumors were measurable after 6 days from injection (mean size: 87 ± 25 mm³) and increased with time. Conversely, subcutaneous injection of DU145/RZ cells resulted in the presence of a palpable nodule in all mice (mean size: 70 ± 27 mm³) after 6 days. However, all these tumors achieved a complete regression by 30 days (Figure 6), and no evidence of tumor regrowth was found during the entire observation period (50 days).

Discussion

Ribozymes are small RNA molecules that possess endonucleolytic activity and catalyse the hydrolysis of specific phosphodiester bonds, resulting in the cleavage of the RNA target sequences. Specifically, hammerhead ribozymes cleave substrate's RNAs at NHH triplets 3' to the second H, where N is any nucleotide and H is any

nucleotide but guanidine (Kore *et al.*, 1998). These catalytic RNA motifs have received much attention in view of their potential usefulness for gene therapy due to their inherent simplicity, small size and ability to be incorporated into a variety of flanking sequence motifs without changing site-specific cleavage capacities. In fact, several studies on experimental human tumor models have demonstrated the feasibility of this approach for the inhibition of a variety of cancer-related genes (Lewin and Hauswirth, 2001).

In the present study, we showed the possibility to reduce markedly the expression of the antiapoptotic factor survivin in two human androgen-independent prostate cancer cell lines by the use of a hammerhead ribozyme targeting exon 1 within survivin mRNA. To obtain a high level of expression of the ribozyme within tumor cells and to target it to the same cellular compartment as its target mRNA substrate, we embedded the ribozyme sequence in the context of the adenoviral *VAl* gene and delivered it to cells through a retroviral vector. The gene is actively transcribed by RNA polymerase III in a variety of cell types, and the resulting transcript is characterized by a cytoplasmic localization (Prislei *et al.*, 1997). Moreover, it has been demonstrated that embedding a ribozyme in the context of this exogenous RNA molecule provides appropriate conformation for catalytic activity and stability (Cagnon and Rossi, 2000).

Ribozyme-mediated downregulation of survivin expression in polyclonal cell populations obtained from both DU145 and PC-3 cell lines resulted in a significant increase in the rate of spontaneous apoptosis, which was accompanied by the proteolytic activation of caspase-9 and caspase-3 and a significant increase in their *in vitro* catalytic activity. These findings corroborate previous evidence indicating that interference with survivin function by the use of antisense oligonucleotides (Chen *et al.*, 2000; Olie *et al.*, 2000) or survivin dominant-negative mutants (Grossman *et al.*, 2001; Mesri *et al.*, 2001) led to an increased apoptotic cell death in different human tumor models; furthermore, they point to a selective role of survivin in antagonizing mitochondrial-dependent apoptosis. In this context, a possible direct interaction of survivin with caspase-9 has been reported by O'Connor *et al.* (2000) whereas more recently Song *et al.* (2003) suggested an alternative model for indirect inhibition of caspases by survivin, which requires Smac/Diablo as intermediate protein. This mitochondrial factor, which is released into the cytosol in response to different apoptotic stimuli, was found to bind to some IAPs (including XIAP, cIAP₁, cIAP₂ and livin), thus preventing them from inhibiting caspases (Du *et al.*, 2000; Vucic *et al.*, 2002). The ability of survivin to interact physically with Smac/Diablo and, as a consequence, sequester it would allow other IAPs to block caspases without being antagonized.

Inhibition of survivin expression also caused an increased apoptotic response of DU145/RZ cells to the anticancer drug cisplatin. This finding extends our previous observation indicating that transfection of JR8 human melanoma cells with plasmid vectors carrying ribozymes targeting exons 1 and 3 in the

survivin mRNA renders them more susceptible to cisplatin-induced apoptosis (Pennati *et al.*, 2002), and is also in keeping with results obtained by Grossman *et al.* (2001) with the survivin Thr34-Ala mutant in a different human melanoma cell line. We also recently demonstrated that ribozyme-mediated inhibition of survivin expression was able to sensitize JR8 melanoma cells to gamma irradiation-induced apoptosis (Pennati *et al.*, 2003). Enhanced chemotherapy-induced cell death has also been reported in a number of experimental studies carried out in human tumor models of different histologies, in which targeting of survivin by means of antisense oligonucleotides, dominant – negative mutants and CDC2 kinase inhibitors – that block CDC2 phosphorylation of survivin on Thr34, which seems to be essential for the cytoprotective function of the protein (O'Connor *et al.*, 2000) – resulted in an increased sensitivity to anticancer agents including etoposide, doxorubicin and taxol (Olie *et al.*, 2000; O'Connor *et al.*, 2002; Wall *et al.*, 2003).

Consistent with a major role of survivin in preserving the mitotic apparatus and allowing normal mitotic progression (Giodini *et al.*, 2002), we found the appearance of a polyploid cell subpopulation in the DU145/RZ cell line as a possible consequence of the almost complete inhibition of survivin expression. Since no evidence of polyploid cells was seen in the PC-3/RZ cell line, in which a larger fraction of residual survivin protein was still present, inhibition below a certain threshold might be insufficient to abrogate survivin function in the control of mitotic progression. Moreover, a significant reduction of the *in vitro* proliferative potential, as indicated by a markedly increased doubling time, was only appreciable in DU145/RZ cells. Microarray analysis carried out in these cells pointed to an altered pattern of expression of genes involved in cell cycle control; decreased expression of some of these genes, notably those that are positive regulators of cell cycle progression such as *CDK2*, *cyclin B1* and *cdc25B*, was confirmed by Western blot analysis.

Regarding the effect of survivin inhibition on the tumorigenic potential of prostate cancer cells, we found that DU145 cells were unable to grow when xenografted in athymic mice. Based on the results obtained in DU145/RZ cells as well as on previous evidence derived from *in vivo* studies (in which downregulation of survivin through the use of survivin Thr34-Ala mutant suppressed *de novo* tumor formation and inhibited the growth of established tumors in immunodeficient mice) (Grossman *et al.*, 2001; Mesri *et al.*, 2001), it could be assumed that the decreased tumorigenic potential of DU145/RZ was due an enhanced spontaneous apoptosis and reduced proliferative potential as a consequence of survivin inhibition.

Overall, the results from this study represent the first evidence that manipulation of the survivin pathway by a ribozyme-mediated approach is able to increase spontaneous and drug-induced apoptosis and decrease the tumorigenic potential of androgen-independent prostate cancer cells. Specifically, the ability to restore the susceptibility to programmed cell death in DU145 cells

that are characterized not only by a high level of expression of antiapoptotic proteins, such as survivin and other IAPs (McEleny *et al.*, 2002), but also by the lack of expression of proapoptotic proteins like Bax (Honda *et al.*, 2001) is particularly relevant. Our data would suggest that in these cells the enhanced apoptotic response is mainly attributable to the almost complete inhibition of survivin, although the concomitant induction of proapoptotic factors like BID (Chao and Korsmeyer, 1998), as indicated by microarray and Western blot analysis, could play an additional role.

Whether selective inhibition of survivin can be used as a therapeutic modality aimed to inhibit the growth and enhance the sensitivity to specific anticancer drugs of androgen-independent prostate cancer is an interesting issue that deserves further investigation. However, since multiple molecular pathways seem to play a role in mediating androgen-independent progression and treatment resistance in this malignancy (Howell, 2000), inhibition of a single target could be insufficient to control adequately tumor growth. In this context, exploration of additive or synergistic effects of combinations of selective inhibitors targeting different cellular pathways in preclinical models of androgen-independent prostate cancer could provide the rational basis for the design of new multitarget clinical therapies.

Materials and methods

Experimental models

The androgen-independent human prostate adenocarcinoma cell lines DU145 and PC-3 were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained as a monolayer in RPMI-1640 (BioWhittaker, Verviers, Belgium) medium containing 10% fetal bovine serum and 0.1% gentamycin in a humidified atmosphere of 5% CO₂ in air.

Synthesis of VAI-fusion ribozymes

A hammerhead ribozyme directed against the 3' end of the CUA₁₁₀ triplet located in exon 1 of survivin mRNA was used in the study (Figure 1a). The ribozyme coding sequence was inserted into the adenoviral VAI RNA. The VAI-fusion ribozyme was obtained as follows: a double-stranded DNA fragment encoding the ribozyme sequence was inserted into the *Bgl*II site of the *pBS-VAI* plasmid (Prislei *et al.*, 1997). The resulting *VAI*-ribozyme cassette was then excised by digestion with *Eco*RI and *Hind*III, blunted, and inserted into the unique *Nhe*I site of *pCLXSN* vector, located in the 3' LTR (Naviaux *et al.*, 1996). The resulting vector was named *pCLXSN/RZ*. A control vector, *pCLXSN/CTR*, was generated by cloning the sequence of an irrelevant ribozyme directed against the FIV primer binding site.

Retroviral vectors and transductions

Transducing retroviral vectors were obtained by cotransfection of HEK293gp packaging cells (constitutively expressing *gag* and *pol* genes) (Somia *et al.*, 2000) with 5 µg of plasmid vectors (*pCLXSN/RZ* or *pCLXSN/CTR*) and 5 µg of *VSV-G*-encoding plasmid by using the calcium phosphate precipitation procedure. After 6 h incubation at 37°C in a 5% CO₂ humidified atmosphere, the transfection cocktail was washed out and replaced with fresh medium. The viral particle-containing

supernatants were collected 24, 48 and 72 h after transfection and used to infect DU145 and PC-3 cells in the presence of polybrene (8 µg/ml). The viral titer of all vector transfectants was estimated to be ~5 × 10⁷ provirus forming units/ml by assessment of the amount of neomycin-resistance transfer on 3T3 mouse fibroblasts in the presence of 0.5 mg/ml G418. The transduced cells were maintained in RPMI medium supplemented with G418 for 1 month and polyclonal cell populations were selected. Cells were harvested, expanded and screened for survivin expression levels. Four polyclonal cell populations, two expressing the antisurvivin ribozyme (DU145/RZ and PC-3/RZ) and two expressing the control ribozyme (DU145/CTR and PC-3/CTR), were selected for the study.

Reverse transcriptase-PCR analysis of ribozyme expression

Total RNA was isolated from parental and infected cells using Trizol (Life Technology Inc., Gaithersburg, MD, USA) and reverse-transcribed using a GeneAmp RNA PCR core kit (Applied Biosystems, Roche Molecular System Inc., Branchburg, NJ, USA) according to the manufacturer's instructions. To analyse ribozyme expression, the resultant cDNA was amplified using VAI sense (5'-GGG CAC TCT TCC GTG GTC-3') and VAI antisense (5'-AAA GGA GCG CTC CCC CGT TG-3') primers (MWG Biotech AG, Ebersberg, Germany), and by performing 30 cycles of PCR (at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min), followed by a 7-min extension step at 72°C. PCR products were verified by agarose gel electrophoresis. The vectors *pCLXSN/RZ* and *pCLXSN/CTR* were used as controls for the correct fragment size during PCR amplification.

Western immunoblotting

Total cellular lysates were separated on a 12% sodium dodecylsulfate-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in phosphate-buffered saline (PBS) with 5% skim milk and incubated overnight with primary antibodies specific for survivin, caspase-9, caspase-3, BID (Abcam Limited, Cambridge, UK), CDK2, CDK4, cyclin B1, cdc25A, cdc25B, E2F-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), tubulin β (Sigma-Aldrich Inc., Saint Louis, MO, USA) and GAS6 (kindly provided by Dr C Schneider, LNCIB, Trieste, Italia). The filters were then incubated with the secondary peroxidase-linked whole antibodies (Amersham Biosciences Europe, Freiburg, Germany). Bound antibodies were detected using the enhanced chemoluminescence Western blotting detection system (Amersham Biosciences). An anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Santa Cruz Biotechnology) was used on each blot to ensure equal loading of protein on the gel. The results were quantified by densitometric analysis using the Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

Flow cytometry

Cells were washed in PBS and stained with a solution containing 50 µg/ml propidium iodide, 50 mg/ml RNase and 0.05% NP40 for 30 min at 4°C and then analysed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). The cell cycle distribution was evaluated on DNA plots using the CellFit software according to the SOBR model (Becton Dickinson).

Apoptosis analysis

For TUNEL analysis, cells were harvested and fixed in 4% paraformaldehyde for 45 min at room temperature. After

rinsing with PBS, the cells were permeabilized in a solution of 0.1% Triton X-100 in sodium 0.1% citrate for 2 min in ice. Samples washed with PBS were then incubated in the TUNEL reaction mixture (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C in the dark, and after rinsing with PBS they were suspended in PBS and analysed by a FACScan flow cytometer (Becton Dickinson). The results were expressed as the percentage of TUNEL-positive cells in the overall cell population.

Caspase-9 and caspase-3 catalytic activity was determined by means of the Caspase-9/Mch6 Fluorometric Protease Assay Kit (MBL, LTD, Japan) and the Caspase-3 Assay Kit (BD Biosciences, Pharmingen, San Diego, CA, USA), respectively. Briefly, total protein and the specific fluorogenic substrate (leu-glu-his-asp-7-amino-4-trifluoromethylcoumarin, LEHD-AFC, for caspase-9 and *N*-acetyl-asp-glu-val-asp-aldehyde-7-amino-4-methylcoumarin, Ac-DEVD-AMC, for caspase-3) were mixed for 1 h at 37°C. In the assay for caspase-3 activity, a negative control was obtained by incubating each sample in the presence of the caspase inhibitor Ac-DEVD-CHO. The hydrolysis of the specific substrates for caspase-9 and caspase-3 was monitored by spectrofluorometry at 505 and 440 nm, respectively.

Analysis of gene expression using oligonucleotide arrays

Total RNA was isolated using Trizol (Life Technologies Inc.) from cells harvested at three different passages of *in vitro* culture, pooled and submitted to microarray analysis using the U133A chip, which contains probes to 23 000 known genes, from Affymetrix (Santa Clara, CA, USA). Biotinylated cRNA probe generation as well as array hybridization, washing and staining was carried out according to the standard Affymetrix GeneChip protocol. Fluorescence intensity for each chip was captured with an Agilent G2500A GeneArray scanner. Absolute analysis of each chip and comparative analysis of samples expressing the antisurvivin ribozyme with control samples was performed using the Affymetrix Microarray Suite software. The mean hybridization signal for each sample was

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set to 100 arbitrary units to normalize the signal values of all the genes on the chip between different samples. A treatment/control ratio of ≥ 2.0 or ≤ 0.5 was chosen as the criterion for induction and repression of gene expression, respectively.

In vivo studies

The experiments were carried out using 10-week-old male athymic Swiss nude mice (Charles Rivers, Calco, Italy). The mice were kept in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale per lo Studio e la Cura dei Tumori (Milan, Italy) according to the United Kingdom Coordinating Committee on Cancer Research Guidelines (Workman *et al.*, 1998). Exponentially growing DU145/CTR and DU145/RZ cells were injected subcutaneously (10^7 cells/mouse) into the right flank of the mice (nine mice/group). The mice were inspected daily to establish the take and the time of tumor appearance. Tumor growth was followed by measuring tumor diameters with a Varnier caliper. Tumor volume was calculated according to the formula: $TV (mm^3) = d^2 \times D/2$ where *d* and *D* are the shortest and longest diameters, respectively.

Statistical analysis

Student's *t* test was used to analyse the differences between control and ribozyme-expressing cells in terms of cell doubling time and *in vitro* catalytic activity of caspase-9 and caspase-3. All tests were two-sided. *P*-values < 0.05 were considered statistically significant.

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