

Antisense-mediated inhibition of survivin, hTERT and VEGF in bladder cancer cells *in vitro* and *in vivo*

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Abstract. Since cancer cells are characterised by multiple genetic alterations the single inhibition of one tumour-associated gene might not be sufficient as a therapeutic strategy. We examined the effects of a combined inhibition of survivin, human telomerase reverse transcriptase (hTERT) and vascular endothelial growth factor (VEGF) with antisense oligodeoxynucleotides (AS-ODNs) and small interfering RNAs (siRNAs) in EJ28 and 5637 bladder cancer (BCa) cells. Following verification of the uptake of intraperitoneally applied fluorescence-labelled AS-ODNs and siRNAs in subcutaneous BCa xenografts, the target-directed constructs were tested as single agents in SCID mice bearing subcutaneous EJ28. Simultaneous inhibition of two of the selected transcripts significantly enhanced cell viability reduction compared to the controls consisting of a target directed construct and an appropriate control construct without any homology to the human genome. The uptake of both antisense inhibitor types in the subcutaneous BCa was achieved even without a carrier. *In vivo* studies with 9 consecutive intraperitoneal injections with 20 mg/kg AS-ODNs or 4.6 mg/kg siRNAs revealed the biocompatibility of both antisense inhibitor types and showed anti-tumoural activity of the AS-ODNs used.

Introduction

Bladder cancer (BCa) is one of the most common cancers with approximately 357,000 new cases and 145,000 deaths from the disease every year worldwide (1). Recurrences of BCa occur in up to 75% of affected patients, and 30-50% of patients with high-risk superficial tumours have an increased risk of progression to invasive cancer (2). Treatment of intermediate and high-risk non-muscle-invasive BCa includes transurethral resection of the tumour and intravesical immunotherapy using Bacillus Calmette-Guérin or chemotherapy (CT) mainly using mitomycin C (MMC).

Besides conventional cancer therapies - including surgery, CT and radiation - several alternative approaches are under investigation, which are supposed to limit tumour progression and recurrence. These strategies aim at reducing the expression of tumour-related genes, for example using antisense-oligodeoxynucleotides (AS-ODNs) or small interfering RNAs (siRNAs). It seems to be reasonable to target genes functioning as central regulators in the control of cell proliferation and cell death, such as the apoptosis inhibitor survivin (SVV), the human telomerase reverse transcriptase (hTERT) and the vascular endothelial growth factor (VEGF). SVV, a member of the inhibitor of apoptosis protein family, has attracted growing attention as potential target for cancer therapy (3). hTERT constitutes the catalytic subunit of human telomerase (4). It contributes to immortalisation of cells by elongation and stabilisation of telomeres. VEGF is the central mediator of physiological and pathological angiogenesis. Alternative splicing of its mRNA results in the expression of at least 9 VEGF isoforms (5), where VEGF₁₆₅ is the biologically most active and essential isoform. All targets described here are selectively over-expressed in the majority of cancers including human BCa (6-8).

It has been shown previously that AS-ODNs or siRNAs (except for the siRNA targeting VEGF which is shown here for the first time) directed at these targets, induced anti-proliferative effects in BCa cell lines confirming the suitability

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Table I. Sequences of ODNs and siRNAs.

Target	ODN	Sequence (5'→3')		Refs.
survivin	ASsvv286	AAGCGCAACCGGACGAATGC		(10)
hTERT	ASt2331	GGTAGAGACGTGGCTCTTGA		(11)
VEGF	ASvegf857	AGGGACCGTGCTGGGTCACC		(9)
-	NS-K1	TAAGCTGTTCTATGTGTT		(11)
Target	siRNA	Sense strand (5'→3')	Antisense strand (5'→3')	Refs.
survivin	siSVV284	GCAUUCGUCCGGUUGCGCUdTdT	AGCGCAACCGGACGAAUGCdTdT	(12)
hTERT	sihTERT3106	GCUCCCAUUUCAUCAGCAAdTdT	UGCUGAUGAAAUGGGAGCdTdT	(20) ^a
VEGF	siVEGF865	CACGGUCCCUCUUGGAAUdTdT	AUCCAAGAGGGACCGUGdCdT	
-	NS-si	UUCUUCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT	

Phosphorothioates within the ODNs are in bold. ODN, oligodeoxynucleotide; NS, nonsense control; refs., references; siRNA, small interfering RNA. ^asihTERT3106 = sihTERT2.

of this monotherapy (9-13). Moreover, a pre-treatment with AS-ODNs targeted at SVV, hTERT or VEGF resulted in the sensitisation of BCa cells to common chemotherapeutics (14-16).

In addition to the function of AS-ODNs and siRNAs as active monotherapy or as sensitising pre-treatment for different CT, the multi-target gene silencing by antisense inhibitors targeting different transcripts at the same time might represent an attractive field of experimental BCa therapy. The aim of the present study is the evaluation of potential additive anti-tumour effects caused by combinations of AS-ODNs or siRNAs against SVV, hTERT and VEGF in BCa cell lines. Furthermore, after validating the uptake of fluorescence-labelled constructs the single antisense inhibitors were tested in subcutaneous BCa xenografts in mice.

Materials and methods

AS-ODNs and siRNAs. Sequences of AS-ODNs (Invitrogen, Karlsruhe, Germany) and siRNAs (Qiagen, Hilden, Germany) targeted at SVV (acc.no. NM_001168), hTERT (acc.no. AF015950) and VEGF (acc.no. M32977) as well as the sequences of the nonsense (NS) control constructs are shown in Table I.

Cell culture and transfections. The human BCa cell lines EJ28 and 5637 were cultured under standard conditions (37°C, humidified atmosphere containing 5% CO₂) without antibiotics. DMEM (4.5 g/l glucose) containing 10% fetal calf serum (FCS), 1% MEM and 1% HEPES was used for EJ28 (all from Invitrogen). 5637 cells were cultured in RPMI-1640 containing 10% FCS.

After seeding in 96-well or 6-well plates and adherence for 72 h, cells were transfected (4 h, 37°C) in serum-free OptiMEM (Invitrogen). The ODNs (500 nM or 2x250 nM in combinations) were transfected using Lipofectin (ratio 1:3

w/w) following the manufacturer's instructions (Invitrogen). Transfections of siRNAs (400 nM or 2x200 nM for combinations) were performed using DOTAP (ratio 1:4 w/w) (Roche, Mannheim, Germany). In combination experiments each of the antisense inhibitors was incubated separately with the transfection reagent before transfection. The combinations containing AS-ODNs or siRNAs against different targets were compared with control combinations consisting of AS-ODN+NS-K1 or siRNA+NS-si, respectively.

Following transfection, cells were washed with PBS and incubated in culture medium for defined time periods. For further analyses cells were harvested by trypsin treatment (0.05% trypsin/0.02% EDTA, 5 min, 37°C). Detached and adherent cells were pooled and analysed together.

Viability and proliferation assays. Cellular viability was examined in quadruplicates 72 or 96 h after transfection using the cell proliferation reagent WST-1 (Roche). Colony formation assays for EJ28 cells were performed by seeding 100 cells 24 h after treatment in triplicates in 6-well plates and incubation for 9-12 days. After Giemsa staining clonogenic survival was determined by counting all macroscopically visible colonies.

Apoptosis detection and cell cycle analysis. Apoptosis was assessed 24 h after transfection by staining cells with Annexin V/propidium iodide and analysed using flow cytometry (Annexin V-FITC Apoptosis Detection Kit I; FACScan, BD Biosciences, Heidelberg, Germany). The CycleTest plus DNA reagent kit (BD Biosciences) was used for cell cycle analysis 24 h after transfection by flow cytometry.

RNA isolation, cDNA synthesis and quantitative PCR. Total RNA was isolated at defined points in time after treatment by Invisorb Spin Cell/Tissue RNA Mini kit (Invitek, Berlin, Germany). The Superscript II reverse transcriptase

Table II. Sequences of primers and probes and the kits used for quantitative PCR.

Target	Sequences of primers and probes (5'→3')
survivin ^a	Primers: for: GAACTGGCCCTTCTTGGAG, rev: AAGTCTGGCTCGTTCTCAGTG Probe: SVV Taq 86 (Roche, Germany: Universal ProbeLibrary probe: #86, cat.no. 04689119001)
hTERT ^{a,d}	Primers: for: TGCAAAGCATTGGAATCAGA, rev: GGAGTCTGGACGTCAGCAG Probe: hTERT Taq 27 (Roche: Universal ProbeLibrary probe: #27, cat.no. 04687582001)
VEGF ^b	Primers: for: GATCGAGTACATCTTCAAGC, rev: CCCACAGGGATTTTCTTGTC Probe: probe: 6FAM-ATCCTGTGTGCCCTGATGCGATGCGGGTXXT-PH ^c
PBGD ^b	Primers: for: GCTGCAACGGCGGAA, rev: CCTGTGGTGGACATAGCAATGATT Probes: FL: TCGCATAACAGACGGACAGTGTGGTG-FL LC: LC-CAACATTGAAAGCCTCGTACCCTGG-PH
TBP ^b	Primer: for: GAATATAATCCCAAGCGGTTTG, rev: ACTTCACATCACAGCTCCCC Probes: FL: TTTCCCAGAACTGAAAATCAGTGCC-FL LC: LC Red640-TGGTTCGTGGCTCTTATCCTCATG-PH

^aLightCycler TaqMan Master (Roche), ^bLightCycler FastStart DNA Master Hybridization Probes (Roche), ^cX = TAMRA, ^dused for quantification of hTERT in tissue samples, for cell culture experiments the LightCycler TeloTAGGG hTERT Quantification Kit (Roche) was used.

(Invitrogen) and random hexamer primers (Amersham Biosciences, Freiburg, Germany) were used for the reverse transcription of 1 µg total RNA into first-strand cDNA according to the manufacturer's instructions. The mRNA expression of the targets and the reference genes PBGD (porphobilinogen deaminase) and TBP (TATA box binding protein) were quantified in independent duplicates using the LightCycler instrument (Roche; Table II).

Quantification of VEGF protein expression. The concentration of secreted VEGF protein in culture medium was measured using the DuoSet ELISA Developmental System kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Statistics. An unpaired Student's t-test was used to compare the results of the combinations containing two target-specific AS-ODNs or siRNAs with those of the respective combinations with NS-K1 or NS-si (*p≤0.05, **p≤0.01 and *** p≤0.001).

Animal studies. Animal studies were conducted at the University of Luebeck or at epo GmbH (Berlin, Germany). Both institutions possess the required approvals for animal handling and experimental procedures.

In vivo uptake of FITC-labelled AS-ODNs. EJ28 cells (1x10⁷) were injected subcutaneously in 4 T-cell-deficient NMRI: nu/nu mice. After 6 weeks, 20 mg/kg FITC-labelled AS-ODNs were applied intraperitoneally. An untreated mouse was used as control. Mice were sacrificed 24, 48 or 72 h after AS-ODN injection. One half of the tissue was cryosectioned, FITC and DAPI counter-staining was examined by fluorescence microscopy. The other half was macerated for flow cytometry.

In vivo uptake of FITC-labelled siRNAs. EJ28 cells (1x10⁷) were injected subcutaneously into 12 CB17 SCID/SCID mice. After 4 weeks, 5 mg/kg FITC-labelled siRNAs or PBS (control) were applied intraperitoneally. Two siRNA treated mice and one control mouse were sacrificed 12, 36, 60 or 84 h after injection, respectively. Tumours, livers and kidneys were collected. One half of the tissue was cryosectioned, the other half was macerated for flow cytometry. FITC and DAPI counter-staining was examined by fluorescence microscopy.

In vivo inhibition studies. EJ28 cells (5x10⁶) were injected subcutaneously into 49 CB17 SCID/SCID mice. Intraperitoneal treatment with 20 mg/kg ODN (ASsv286, ASst2331, NS-K1) or 4.6 mg/kg siRNA (siSVV284, sihTERT3106, siVEGF865 and NS-si) started one day after tumour cell implantation with 7 mice per group. Constructs were applied 3 times per week for 3 weeks. Mouse weight and tumour volume were examined every 2 or 3 days. One mouse of each group was sacrificed 24 h after the eighth administration, others when tumour volume exceeded 500 mm³ (the earliest 39 days after treatment start). Tumours, livers and kidneys were collected. One half of the tissue was snap-frozen, cryosectioned and used for mRNA expression analyses, the other half was embedded in paraffin and stained with HE or immunohistochemically for SVV, hTERT, VEGF and CD31 (vascular marker). HE slides were examined by an experienced pathologist (M.T.).

Immunohistochemistry (IHC). Paraffin sections of tumours (4 µm) were deparaffinised and rehydrated. Antigen accessibility was achieved by heating samples for 15 min at 121°C in unmasking buffer (Table III). Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in 0.05 M TRIS/0.15 M NaCl buffer pH 7.6 for 10 min. IHC was

Table III. Antibodies and unmasking buffers for immunohistochemistry.

Protein	Antibody	Unmasking buffer
survivin	Rabbit polyclonal antibody to survivin (NB500-201, Novus Biologicals, Littleton, USA)	0.01 M citrate buffer pH 6.0
hTERT	Telomerase (Catalytic Unit) antibody (clone 44F12, Novocastra, Newcastle upon Tyne, UK)	0.01 M citrate buffer pH 6.0
VEGF	Mouse monoclonal (VG1) antibody to VEGF (NB100-664, Novus Biologicals)	0.01 M TRIS/0.001 M EDTA buffer pH 8.0
CD31	Monoclonal mouse anti-human CD31 (clone JC70A, Dako, Hamburg, Germany)	0.01 M TRIS/0.001 M EDTA buffer pH 9.0

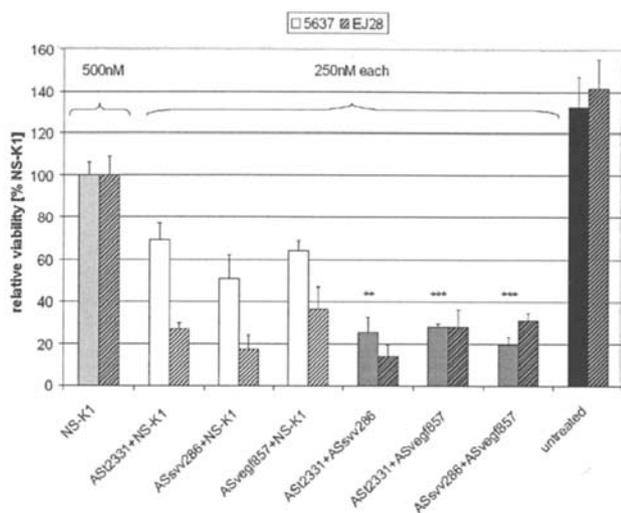


Figure 1. Cellular viability of 5637 and EJ28 BCa cells after transfection with AS-ODNs against SVV, hTERT or VEGF in combination with the NS-K1 control or with each other 72 h after transfection. Data represent means of 5 repetitions with standard deviations. All values are normalised to NS-K1 (100%). Asterisks indicate significant differences compared to NS-K1 + target-specific AS-ODN (** $p < 0.01$, *** $p < 0.001$).

performed using Vectastain Universal Elite ABC kit (Vector; Burlingame, USA) according to the manufacturer's instructions. Primary antibodies (Table III) were incubated at 4°C overnight. Diaminobenzidine was used as peroxidase substrate. Nuclei counterstaining was performed with hemalaun.

Results

Combination of AS-ODNs targeted at SVV, hTERT and VEGF. The AS-ODNs (Table I) were shown previously to downregulate their respective targets and to effectively inhibit the proliferation of BCa cells *in vitro* (9-11). In the present study, each two of the AS-ODNs were combined and delivered simultaneously into different BCa cell lines to examine their effects on cell growth.

Fig. 1 shows the cellular viability of 5637 and EJ28 cells 72 h after transfection with AS-ODNs directed at

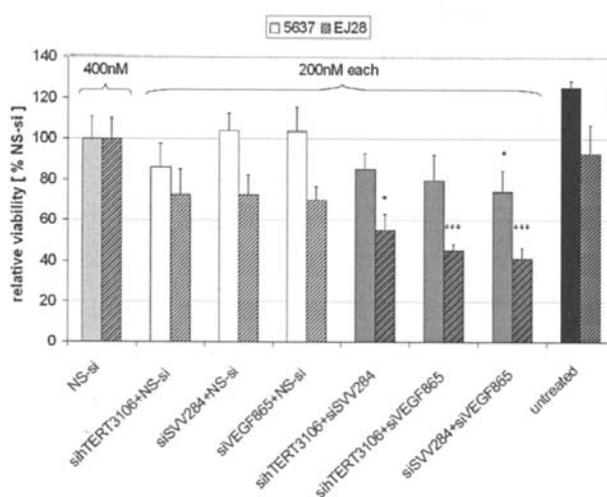


Figure 2. Cellular viability of 5637 and EJ28 BCa cells after transfection with siRNAs against SVV, hTERT or VEGF in combination with the NS-si control or with each other 72 h after transfection. Data represent means of 5 repetitions with standard deviations. All values are normalised to NS-si (100%). Asterisks indicate significant differences compared to NS-si + target-specific siRNA (* $p < 0.05$, *** $p < 0.001$).

SVV, hTERT or VEGF in combination with NS-K1 or in combination with each other. In 5637 cells the combination of each AS-ODN with NS-K1 decreased the viability to 69% (ASSt2331), 51% (ASsvv286) and 64% (ASvegf857) of the NS-K1 control, respectively. The viability inhibition was significantly enhanced by the combinations containing ASSt2331+ASsvv286 (26%), ASSt2331+ASvegf857 (28%) and ASsvv286+ASvegf857 (20%). In EJ28 cells the already strong inhibition of viability by individual AS-ODNs was not enhanced by combining two target-specific constructs.

Cell cycle distribution, decrease in cell counts (by 24-48%) as well as in clonogenic survival (by 34% at most) after transfections with AS-ODN+NS-K1 or with combinations of AS-ODNs were similar. Moreover, apoptosis induction from 12-13% in NS-K1 treated cells to 24-29% in the combinations was not enhanced by the simultaneous inhibition of different targets in comparison to the respective AS-ODN+NS-K1 treatments.

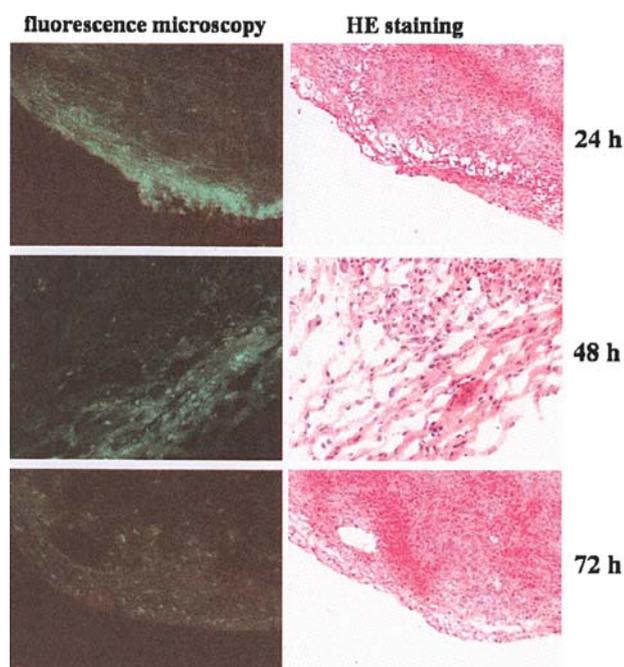


Figure 3. Fluorescence microscopy (magnification: 24 and 72 h, x100; 48 h, x200) and HE staining of subcutaneously xenotransplanted EJ28 cells in NMRI:nu/nu mice after treatment with 20 mg/kg FITC-labelled AS-ODN 24-72 h after intraperitoneal injection.

Design and effects of siVEGF865. SiVEGF865 was designed to target the same secondary structure motif within the VEGF165 mRNA as ASveg857. The construct decreased

the number of VEGF transcripts time-dependently with most prominent effects at 72 h. At this time point, the expression of the VEGF mRNA was reduced to 22-34% in EJ28 and 5637 cells. Furthermore, the VEGF protein content was decreased in both cell lines to at least 50%. Neither significant changes of apoptotic rates nor cell cycle alterations were observed (data not shown). Although siVEGF865 alone was not able to reduce cell viability it significantly sensitised both cell lines to a subsequent chemotherapy with MMC or cisplatin (data not shown).

Combination of siRNAs targeted at SVV, hTERT and VEGF. All combinations of target-directed siRNAs caused 72 h after transfection a significantly enhanced reduction of viability in EJ28 cells compared to the control combinations using the appropriate siRNA+NS-si (Fig. 2). For example, the combination of siSVV284+siVEGF865 decreased cell viability to 41% in comparison to 70 or 73% caused by the control combinations containing siVEGF865+NS-si or siSVV284+NS-si, respectively. However, the effects on viability differed among the tested cell lines. In 5637 cells, all combinations of target-directed siRNAs induced a slightly more potent reduction of viability in comparison to the control combinations with NS-si, whereby only the combination siVEGF865+siSVV284 reached significance (Fig. 2).

In vivo uptake of FITC-labelled AS-ODNs. Fluorescence microscopy demonstrated the uptake of intraperitoneally injected FITC-labelled AS-ODNs in the peripheral areas of the tumours (Fig. 3). Fluorescence signals were most

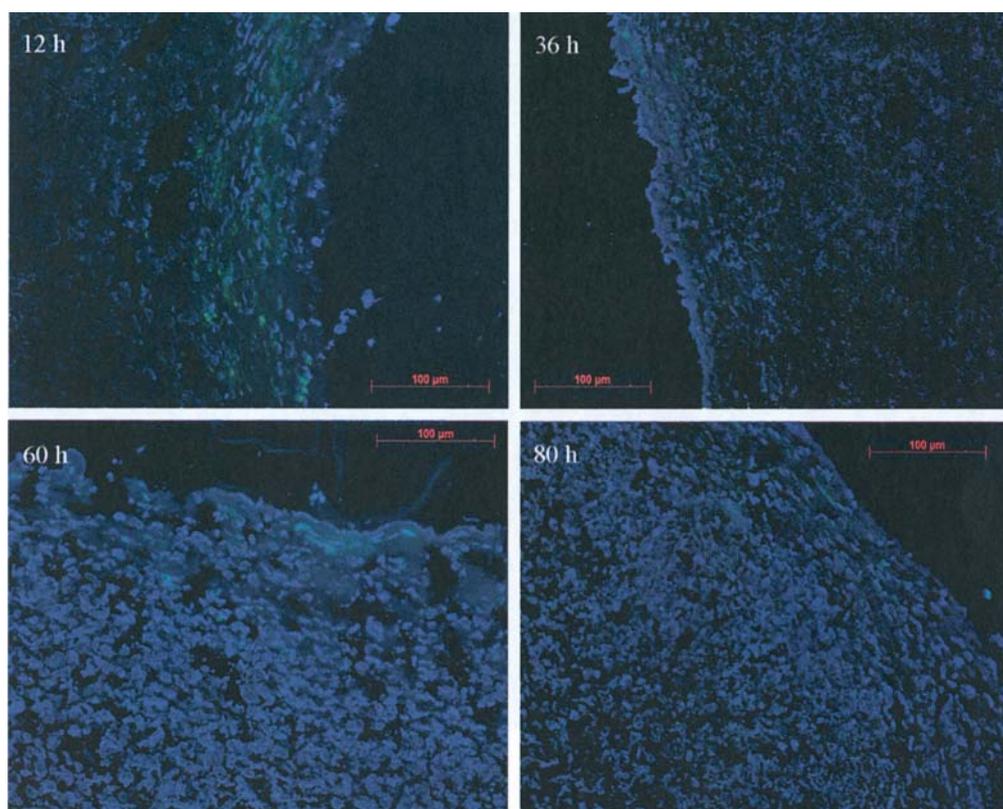


Figure 4. Fluorescence microscopy (magnification, x200) of subcutaneously xenotransplanted EJ28 cells in SCID mice after treatment with 5 mg/kg FITC-labelled siRNA 12-80 h after intraperitoneal injection. Green, FITC-fluorescence; Blue, DAPI nuclei staining.

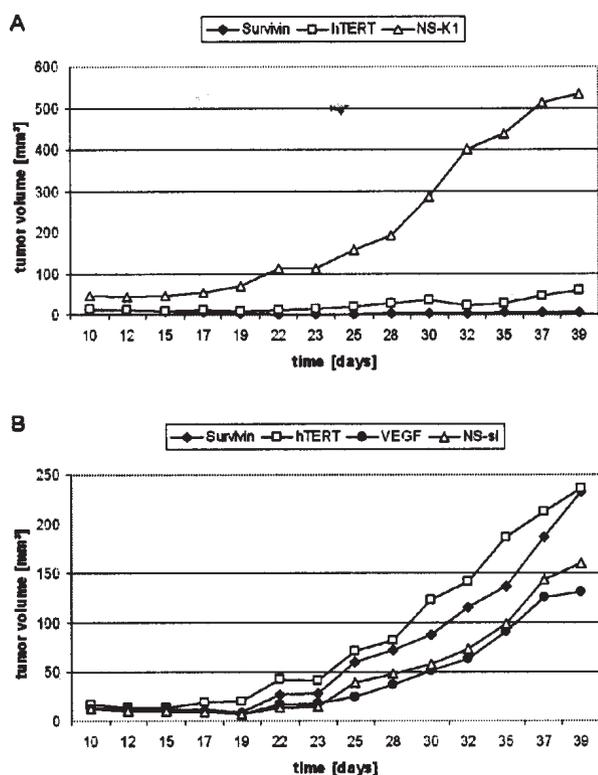


Figure 5. Changes of the tumour volume after intraperitoneal ODN (A) or siRNA (B) treatment of subcutaneously injected EJ28 cells in SCID mice. Mice were treated 3 times a week for 3 weeks with 20 mg/kg ODN or 4.6 mg/kg siRNA. Data points show mean values of treatment groups each consisting of 7 mice.

intense 24 h after treatment but still detectable after 72 h. Flow cytometric analyses revealed AS-ODN uptake in 48% of the tumour cells 24 h after treatment and showed a time-related decrease (24% after 48 h, 10% after 72 h). No FITC fluorescence signals were detected in the control mouse.

In vivo uptake of FITC-labelled siRNAs. Fig. 4 shows the uptake of FITC-labelled siRNAs in the peripheral areas of the tumours. A maximum penetration of about 10 cell layers was detected 12 h after injection. Investigation of the other time points exhibited a time-related decrease of the FITC fluorescence intensity in the tumours. FACS analyses of tissue specimens taken from tumours, livers and kidneys after siRNA treatment revealed only weak FITC fluorescence signals in the macerated tissues (data not shown). No fluorescence was detected in the PBS-treated controls.

In vivo application of AS-ODNs and siRNAs. In this first *in vivo* study with mice bearing EJ28 tumours the intraperitoneal application of AS-ODNs targeting SVV or hTERT significantly reduced tumour cell growth compared to the control (Fig. 5A). In contrast, the injection of siRNAs directed at SVV, hTERT or VEGF did not influence cancer growth (Fig. 5B). Treatment with both antisense inhibitor types showed until 39 days after the first application no significant toxicities, for example the body weight of the mice was not influenced. Furthermore, examined livers and kidneys showed

no evidence of fibrosis, cholestasis or necrosis (data not shown). QPCR analyses of the targets' mRNA expression in tumours indicated a reduction of SVV mRNA after treatment with siSVV284, but not with ASsvv286 directly after the eighth administration as well as after the tumour-size dependent sacrifice (data not shown). For VEGF and hTERT no changes in mRNA expression were found (data not shown). IHC revealed no significant changes of the targets' protein expression (data not shown). No or only marginal vascularisation was observed after CD31 staining in the examined tumours (data not shown).

Discussion

A suitable target for nucleic acid-based inhibition is characterised by its essential function and its selective (over)-expression in tumour cells compared to non-malignant cells. This was previously shown for SVV, VEGF and for hTERT in BCa (10,11,16). Efficiency of ODN and siRNA uptake into BCa cell lines with fluorescence-labelled constructs and the appropriate lipids was previously proven by fluorescence microscopy and flow cytometry. For ODN+Lipofectin an efficient uptake with 99% fluorescence-positive cells was shown already 1 h after transfection (11). For siRNA+DOTAP uptake rate was approximately 95% (unpublished data). The potency of AS-ODNs, which were designed to inhibit the expression of the tumour-related genes SVV, VEGF and hTERT, to repress the viability of different BCa cell lines was also shown previously (9-11). The present study addressed the question of whether combinations of AS-ODNs, which reduce the expression of the selected targets simultaneously, may enhance the inhibition of cellular growth in comparison to appropriate single AS-ODN constructs.

The investigated AS-ODNs targeting SVV, VEGF or hTERT, respectively, clearly decreased the viability of the tested BCa cell lines. The combinations of each two of the different AS-ODNs induced additive effects on cellular viability in 5637 cells but not in EJ28. Since treatments with target-specific AS-ODNs in EJ28 provoked a stronger viability reduction than in 5637, a further enhancement by the combinations was possibly not feasible. Furthermore, the enhanced sensitivity of EJ28 cells compared to 5637 cells might be due to the different genetic background of the cell lines (17). Only few studies have previously described a combined targeting of different transcripts by AS-ODNs. Opalinska *et al* (18) found that the simultaneous inhibition of two oncogenes (MYB and VAV1) in a chronic myelogenous leukemia cell line significantly diminished colony formation (~20% enhancement) compared to the attack of only one of these transcripts. Elez *et al* (19) reported the systemic delivery of two AS-ODNs directed at the regulator of cell cycle progression PLK1 and the apoptosis inhibitor BCL2 into nude mice bearing different human xenograft tumours. The sequential application of these different AS-ODNs induced a strong synergistic effect, which caused the eradication of nearly all tumours after one month of combination therapy.

To compare the effects of different classes of antisense inhibitors siRNAs targeting the same transcripts were used.

The siRNAs directed at SVV (siSVV284) and hTERT (sihTERT3106 = sihTERT2) were used previously (12,20), siVEGF865 is described herein for the first time. Despite the effective downregulation of the VEGF expression by siVEGF865, no direct antiproliferative effects were observed. Similar observations were reported by Yin *et al* (21) who found no inhibition of viability by a VEGF-directed siRNA in melanoma cells. A direct impairment of tumour cell proliferation after downregulation of VEGF would occur in consequence of the inhibition of the autocrine growth stimulation via VEGF receptor signalling in BCa cells (22). Possibly, the reduction of VEGF expression in tumour cells provokes remarkable anti-tumour effects particularly in animal models by the inhibition of neoangiogenesis as reported by Takei *et al* (23). However, in our study siVEGF865 failed to reduce tumour growth *in vivo* (Fig. 5) which might be due to the low level of vascularisation observed.

The *in vitro* inhibition of each two of the three targets by simultaneous application of the respective siRNA constructs showed enhanced anti-proliferative effects in the selected BCa cell lines. In comparison to the control treatments with siRNA+NS-si, all combinations of target-directed siRNAs caused a significantly decreased viability of EJ28 cells, whereas in 5637 cells a significant inhibition was only observed for the combination of siSVV284+siVEGF865 (Fig. 2). A similar approach applying siRNAs targeted at different tumour-related genes was reported by Yin *et al* (21). These authors simultaneously inhibited BCL2, CDK2, HRAS, MDM2 and PRKCA in different tumour cell lines. The clear impairment of tumour cell proliferation after treatment with single siRNA could be enhanced by the combined knock-down of all five genes, which are all involved in pathways essential for tumour growth. These data emphasise the rationality of such multi-target inhibition approaches. However, it is not likely to cause complete cell death due to the complex nature of tumour-related cell signalling (21). Therefore, a combination of simultaneous down-regulation of tumour-related genes with cytotoxic agents is supposed to reach a maximum impairment of tumour cell proliferation.

In contrast to Urban-Klein *et al* (24) but in agreement with Filleur *et al* (25) the present *in vivo* uptake studies with FITC-labelled AS-ODNs and siRNAs show that intraperitoneally applied constructs penetrated the peripheral layers of subcutaneous tumours even without a carrier (Figs. 3 and 4). However, stability and the retention period of the antisense inhibitors, particularly the siRNAs, in the tumour cells should be enhanced to increase the inhibiting efficiency. Therefore, construct backbone modifications as well as complexation of constructs with non-toxic transfection agents like polyethyleneimine (PEI) should be tested (26).

The application of AS-ODNs and siRNAs targeting SVV, hTERT or VEGF revealed the biocompatibility of the constructs. Furthermore, the AS-ODNs but not the siRNAs used showed significant antiproliferative effects which might be caused, at least in part, by non-target specific effects. Inducing off-target effects and stimulating innate immune response is described for both AS-ODNs and siRNAs

(27,28). However, application of siRNAs seems to be safer since their mechanism of action is natural RNA interference.

The combined antisense mediated inhibition of tumour promoting genes represents a promising strategy for cancer treatment. The present study showed in general the *in vivo* safety and applicability of different antisense inhibitor types. Furthermore, the anticancer activity of the AS-ODNs used is demonstrated.

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