In Vivo Studies on the Availability and Toxicity of Antisense Oligonucleotides in Bladder Cancer

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Abstract. *Background: The urinary bladder is an ideal organ for topical treatment. A substantial number of bladder cancer patients are resistant to conventional intravesical therapy. In search of new agents, antisense oligonucleotides (AS-ON) may be interesting candidates. The availability and toxicity as well as the effectivity of AS-ON after intravesical instillation in different rodent models were examined. Materials and Methods: Acute toxicity of AS-ON was tested by intravenous application (215-1,000 mg/kg body weight (bw)) in NMRI mice (n=30). The uptake and distribution of isotope-labelled AS-ON in bladder tissue was determined in Sprague Dawley rats (n=12) by radioactivity after intravesical application (2.5 mg/kg bw ^{3}H-labelled AS-ON). Additionally, uptake and effectivity studies of AS-ON in tumors were performed in MB-49 bladder cancer-bearing C57/Bl6 mice (n=6) by immunohistochemistry and fluorescence microscopy. Results: No systematic side-effects were noticed after intravenous application of physiological doses of AS-ON in NMRI mice. The mortality rate was 20% at the highest dose of 1,000 mg/kg bw. The highest AS-ON availability after intravesical application in rats was noticed in the bladder wall (12.3 μg/g), while the systemic concentration was low (1.1 μg/g). In fluorescence microscopy analysis, AS-ON were detected in the outer cells of the bladder wall and around vessels. AS-ON accumulated in the cytoplasm and in the nuclei. Immunohistochemical analysis demonstrated a reduction of the Ki-67 positivity after treatment with AS-ON (43%) compared to the untreated controls (58%). Conclusion: These preclinical experiments have shown that intravesical antisense oligonucleotides are safe and accumulate in the bladder and in bladder tumors, whereas systemic concentrations remain low. These data are the basis of a first clinical phase I study with intravesical instillation of Ki-67 antisense oligonucleotides.*

More than 90% of bladder carcinomas are transitional cell carcinoma; the remainder are squamous cell carcinoma or adenocarcinoma. Bladder cancer is the second most common urologic malignancy in the Western worlds with an estimated 67,160 new cases diagnosed each year in the United States (1) and 25,950 in Germany (2).

The bladder has three main histological layers: the urothelium, the suburothelial layer of loose connective tissue, called the lamina propria, and the detrusor muscle. Seventy to eighty percent of patients have a non muscle-invasive bladder cancer (*e.g.* confined to the mucosa=Ta, T1, Cis according to the 2002 TNM classification). Principally, treatment modalities differ strongly between non muscle-invasive tumors and detrusor-invasive tumors. The mainstay of treatment for non muscle-invasive bladder has always been complete transurethral resection (TUR), first control cystoscopy after 3 months and further cystoscopic follow-up surveillance. However, overall, more than 50% of patients diagnosed with non muscle-invasive bladder cancer experience a recurrence of their tumor within one year (3). The probability of recurrences at one year after TUR depends on tumor characterictics and ranges from 15% to 70% (4). The probability of progression to muscle-invasive disease at five years ranges from about 7% to 40% (5). Consequently, as an adjunct to tumor resection, intravesical chemotherapy or intravesical immunotherapy are routinely used to reduce tumor recurrence rates and possibly delay or prevent tumor progression to muscle-invasive disease.

Since the urinary bladder is an ideal organ for local topical treament, numerous agents have been instilled intravesically in order to decrease recurrence and eventually progression of non muscle-invasive tumors. Nowadays, intravesical chemotherapy with agents such as mitomycin C, epirubicin or doxorubicin, as well as immunotherapy

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with Bacillus Calmette Guerin (BCG), is recommended as an adjuvant treatment after TUR in dependence of the recurrence and progression risk. Intravesical BCG and chemotherapy result overall in a 30-80% reduction of tumor recurrence compared to TUR alone and a significant prolongation of the recurrence-free interval (6). Furthermore, BCG can reduce tumor progression when maintenance therapy is given (7, 8).

However, instillation of BCG and chemotherapy is associated with typical local and systemic side-effects. Additionally, approximately one third of patients are resistant to therapy. Thus, new more effective agents such as antisense constructs are needed especially in these patients.

Antisense oligonucleotides (AS-ON) against different target genes are currently evaluated in numerous oncological trials, with subcutaneous, intraperitoneal, intravenous, and intravesical routes of administration (9). Targeting of various tumor-associated genes has shown promising preclinical and clinical activity of AS-ON in cancer (10-12). Clinical trials using AS-ON given intravenously have shown good toleration, with initial fever and chills being the most common side-effects. Other reported side-effects include hypotension, liver dysfunction, and myelosupression. Most adverse effects are either transient or reversible with discontinuation of therapy. Importantly, effective dosing of AS-ON is significantly lower than the maximum tolerated dose and is well below the dose at which a majority of the adverse effects occur (13). Intravesical therapy allows direct contact between the tumor and antineoplastic agents. High local efficacy of these compounds after topical treatment has been described but dosage, toxicity and biological side-effects of AS-ON administrated intravesically are yet unknown (14, 15).

In this study, we examined the intravesical availability of AS-ON in different mouse and rat models. Ki-67 was chosen as the target gene.

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent in resting cells (G(0)), makes it an excellent marker for cell proliferation (16). Although the Ki-67 protein is well characterized at the molecular level and extensively used as a proliferation marker, its functional significance still remains widely unclear. Since proliferation is highly associated with malignant transformation, down-regulation of this antigen may represent a promising strategy for cancer therapy. Previous *in vitro* and *in vivo* studies in different tumor models with Ki-67 antisense constructs demonstrated significant antitumoral effects (17, 18).

Materials and Methods

Cell lines. The murine bladder cancer cell line MB-49 was used to establish the orthotopic bladder cancer model. Cells were grown under standard conditions in an incubator with 5% CO_2 in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units per ml penicillin and 100 μg/ml streptomycin (PAA, Cölbe, Germany).

Oligonucleotides. Complete Phosphorothioate Ki-67 directed AS-ON were used (in part 3'FITC-labeled) with the sequence: 5'-ACC AGG TGA GCC GAG GAC GCC AT-3'(Eurogentec, Seraing, Belgium).

Immunohistochemistry. Frozen sections were cut to 4 μm-sections and placed on slides which were dried at RT for 3 hours before staining. Fluorescence microscopy was performed with an appropriate filter at a magnification of ×63.

Immunohistochemical staining of the Ki-67 antigen was performed with the Tec3 antibody as previously described (19). Briefly, primary Tec3 antibody (1:50) (DAKO, Hamburg, Germany), secondary peroxidase-labelled rabbit anti rat (1:2,500) (Dianova, Hamburg, Germany) and tertiary peroxidase-labelled anti-rabbit (1:50) (Jackson Immuno Research, Hamburg, Germany) were used. For evaluation of antibody-positive fractions at least 100 cells were counted in three different regions and the mean number of all counts was determined.

Animal experiments. NMRI mice: 15 male and 15 female NMRI mice, (Charles River, Sulzfeld, Germany) weighing 18-23 g were housed in groups of 5 animals in Makrolon cages (type III) at a room temperature of 22±3°C and a relative humidity of 55±15%. Three dose levels were tested: 215 mg, 464 mg and 1,000 mg AS-ON/kg b.w. Groups for each level consisted of 10 mice (5 female, 5 male) and were examined for toxicity over a 14-day observation time. Application was intravenous as bolus injection into a tail vein, 15 s/dose in 20 ml/kg b.w. NaCl 0.9%. After observation, all mice were sacrificed and dissected.

Sprague Dawley rats: Twelve 9- to 10-week-old female Sprague Dawley rats (Forschungsinstitut fuer Versuchstierzucht, Himberg, Germany) were single housed per cage in Makrolon cages III at a mean room temperature of 22±1°C and a humidity of 70±10% with free access to food and water.

Rats were anaesthetized with urethane (4 ml/kg b.w.). A polyurethane 0.4 mm O.D. ×8 cm (Luther Medical Products, CA, USA) catheter was set and 0.1 ml of aqueous, ³H-labeled, AS-ON at 5 mg/ml was administered into the bladder. The urethra was ligated at its orifice for 2 hours. At the end of the exposure time the radioactivity was determined in bladder, content of the bladder plus rinsing, carcass and blood plasma in 6 rats. Aliquots were digested with Soluene 350 and then incorporated into the liquid scintillator Ultimagold MV (Packard BioScience, Dreieich, Germany). ³H was determined in a liquid scintillation counter (Tri-Carb 2200CA, Packard) with automatic external standardization and facilities to substract background counting rates and to compute quench corrected decay rates.

The remaining 6 rats were sacrificed and dissected and the bladders were evaluated histopathologically. Samples of the bladders were deep frozen and sectioned in a Cryo-Cut microtome. Freshly obtained sections were vapour fixed with formaldehydes and air

Table I. *Toxicity after single intravenous application of AS-ON at increasing doses in NMRI mice.*

Ki-67 AS-ON systemically	215 mg/kg n=10	464 mg/kg n=10	1000 mg/kg n=10
Mortality	-	-	2/10
Reduction of motility	-	10/10	10/10
Dyspnea	-	6/10	10/10
Ataxia	-	8/10	10/10
Tonic convulsion	-	-	1/10
Body weight changes	-	-	-

Observation time, t=14 days; 5 female and 5 male mice per group.

Figure 1. *Availability of AS-ON in Sprague Dawley rats after intravesical application of ^{3}H-labeled AS-ON for 2 hours. Concentration of ^{3}H-labeled AS-ON measured in µg equivalents/g. AS-ON at 2,5 mg/kg b.w. was applied to each rat in the treatment group, n=6 female rats. Mean values are shown.*

dried. All experiments were approved by the Institutional Board and by the local Ministry of Environment, Nature and Forestry Germany.

C57/Bl6 mice: Six 6- to 8-week-old female C57/Bl6 mice (Charles River, Sulzfeld, Germany) weighing approximately 17 g were maintained at the animal care facility for 2 weeks before use. Mice were housed 5 per cage in a limited access area at a mean room temperature of 20±1°C and a humidity of 50±10% with free access to food and water. The experiments were approved by the Institutional Board and by the Ministry of Environment, Nature and Forestry of Schleswig-Holstein, Germany.

Intravesical tumor implantation was performed as described elsewhere (20). Briefly, after anaesthesia with pentobarbital *i.p.* 0.06 mg/g, shaving of an 1 cm^2 area at the backs was performed and the mice were catheterized. For this purpose, a 24-gauge teflon intravenous catheter (Insyte-W®; Becton Dickinson) was inserted intravesically using a lubricant (Instilla Gel®; Farco-Pharma, Köln, Germany). Mice were placed on their backs on the ground plate of the cautery unit. To optimize contact, an ECG-electrode contact gel was used. A guide wire was inserted into the bladder through the catheter and attached to the cautery unit. A monopolar coagulation was applied for 5 seconds at the lowest coagulation setting. After removal of the guide wire, the tumor cell suspension (1×10^4 MB 49 tumor cells in 50 µl RPMI 1640) was instilled. The catheters were clamped off immediately after instillation with a clamp and left in place until awakening.

Thirty days after tumor implantation the mice were anaesthetized again with pentobarbital *i.p.* 0.06 mg/g. The mice were catheterized and the FITC-labeled antisense oligonucleotides were applied to the bladder. All mice received 100 µl suspension. Two mice received 100 µl 0.9% NaCl, 25 µg (1 µg/ml) FITC-labeled antisense oligonucleotides, or 50 µg (2 µg/ml) each. The mice were sacrificed 12 hours after treatment. Bladders and kidneys were removed and either immediately stored in liquid nitrogen or fixed in formaldehyde.

Results

For evaluation of toxicity, single intravenous application of the AS-ON at increasing doses was performed in NMRI mice. Following administration of the low dose level (215 mg/kg b.w.), mice did not show clinical signs of toxicity at any time point (5, 15, 30, 60 min, 3, 6 and 24 hours and daily until the end of observation at day 14). No influence of animal behaviour or premature mortality was noted. A single intravenous bolus of
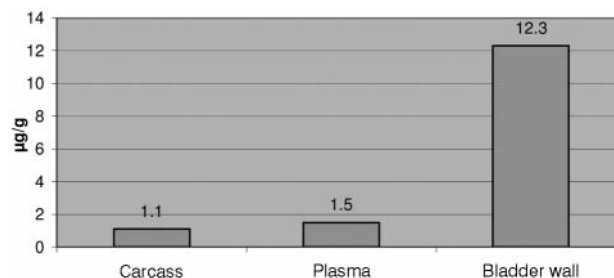
464 mg/kg b.w. led to slightly reduced motility in all animals, slight ataxia in 4 out of 5 animals female and male, slight dyspnea in 4 out of 5 male and 2 out of 5 female animals (Table I). No premature mortality was noticed. After administration of 1,000 mg/kg b.w., reduced motility, ataxia and dyspnea occurred in all animals. After administration, 3 out of 5 male and 4 out of 5 female mice moved to an abdominal position; a lateral position was noticed in 2 out of 5 animals, slight tremor occurred in 1 out of 5 female animals and tonic convulsions in 1 out of 5 male animals. One of 5 male mice died within 24 hours after administration, while 1 out of 5 female animals was found dead at day three. No change in body weight of any surviving animals was noted. No macroscopic changes were found at necropsy.

The distribution of ^{3}H-labeled AS-ON in the body was examined in rats. The rats were anaesthetised, a catheter was set and the test solution was administered to the urinary bladder. The urethra was ligated and kept so for 2 hours. At the end of the exposure, rats were sacrificed and evaluated. Intravesical application was well tolerated. At necroscopy no macroscopic findings were detected. Histopathological findings in the bladders of the rats were edema of the submucosa and dilatation of the veins. The mean total recovery of the applied radioactivity was 90.4%. About half (49.9%) of the applied ^{3}H-labeled AS-ON was recovered in the content of the bladder. A low amount (0.46%) was detected in the bladder itself. The penetration through the bladder wall amounted to 40.1% of the applied dose. The highest concentration of the AS-ON was measured in the bladder wall (12.3 µg equivalents/g), whereas the mean concentration in the plasma of 1.5 µg equivalents/g matched the concentration in the carcass (1.1 µg equivalents/g) (Figure 1). Accumulation of the AS-ON was found focally in isolated areas of the epithelium of the mucosa (predominantly intracellular). Additionally, a diffuse distribution in the outer muscular layers was seen (predominantly intercellular). All other structures of the bladders were free of notable accumulations.
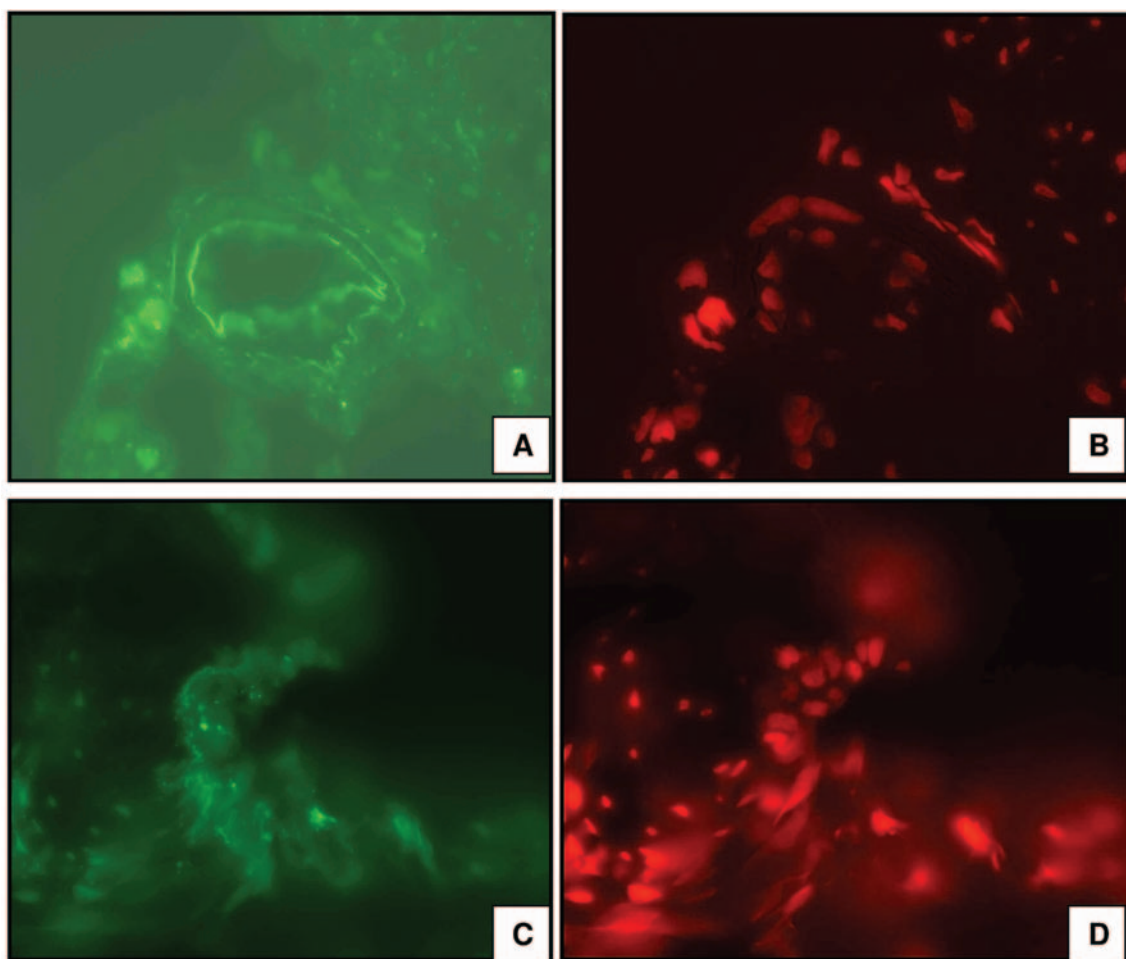
Figure 2. *Instillation of 50 μg Ki-67 AS-ON: two representative areas are shown (A/B and C/D) (×63), nuclei were counterstained with propidium iodide (red); AS-ON were FITC-labeled (green). A/B, bladder. C/D, bladder wall and endothelium.*

Furthermore, the availability of Ki-67 directed AS-ON coupled with FITC was studied after intravesical instillation in an orthotopic bladder tumor model. Twelve hours after instillation of two different concentrations of AS-ON (25 μg and 50 μg/mouse) mice were sacrificed. The bladders and kidneys were excised and frozen sections were prepared. To determine the cellular distribution of FITC-labelled AS-ON the excised bladder and kidneys were counterstained with propidium-iodide and evaluated by fluorescence microscopy. FITC staining was detected in the outer cells of the bladder wall and in/around the vessels (Figure 2). Staining was noted in the nuclei and in the cytoplasm (Figure 2). No FITC fluorescence was found in the kidneys.

In further immunohistochemistry studies, sections of the tumor-bearing bladder walls were investigated with Ki-67 antibodies. The treated mice showed a reduced Ki-67 positivity (43%) compared to the sections from untreated mice (58%) (Figure 3).

Discussion

Antisense oligonucleotides are effective tools to reduce any target gene expression in a sequence-specific manner. In previous studies, comparable systemic delivery of oligonucleotides has been achieved by subcutaneous, intraperitoneal and intravenous application (21). Despite the existence of several ongoing or finished oncological clinical phase III studies, the intravesical availability of antisense oligonucleotides remains largely unknown. In this study, the systemic toxicity, the intravesical uptake and effectivity of Ki-67 directed AS-ON were investigated.

The target gene down-regulation and thus the postulated therapeutic molecular effect can only be detected when an effective AS-ON uptake is achieved. The intracellular incorporation of unmodified oligonucleotides is poor since they are degraded by nucleases in plasma with a half-life of about 5 minutes after intravenous injection in different
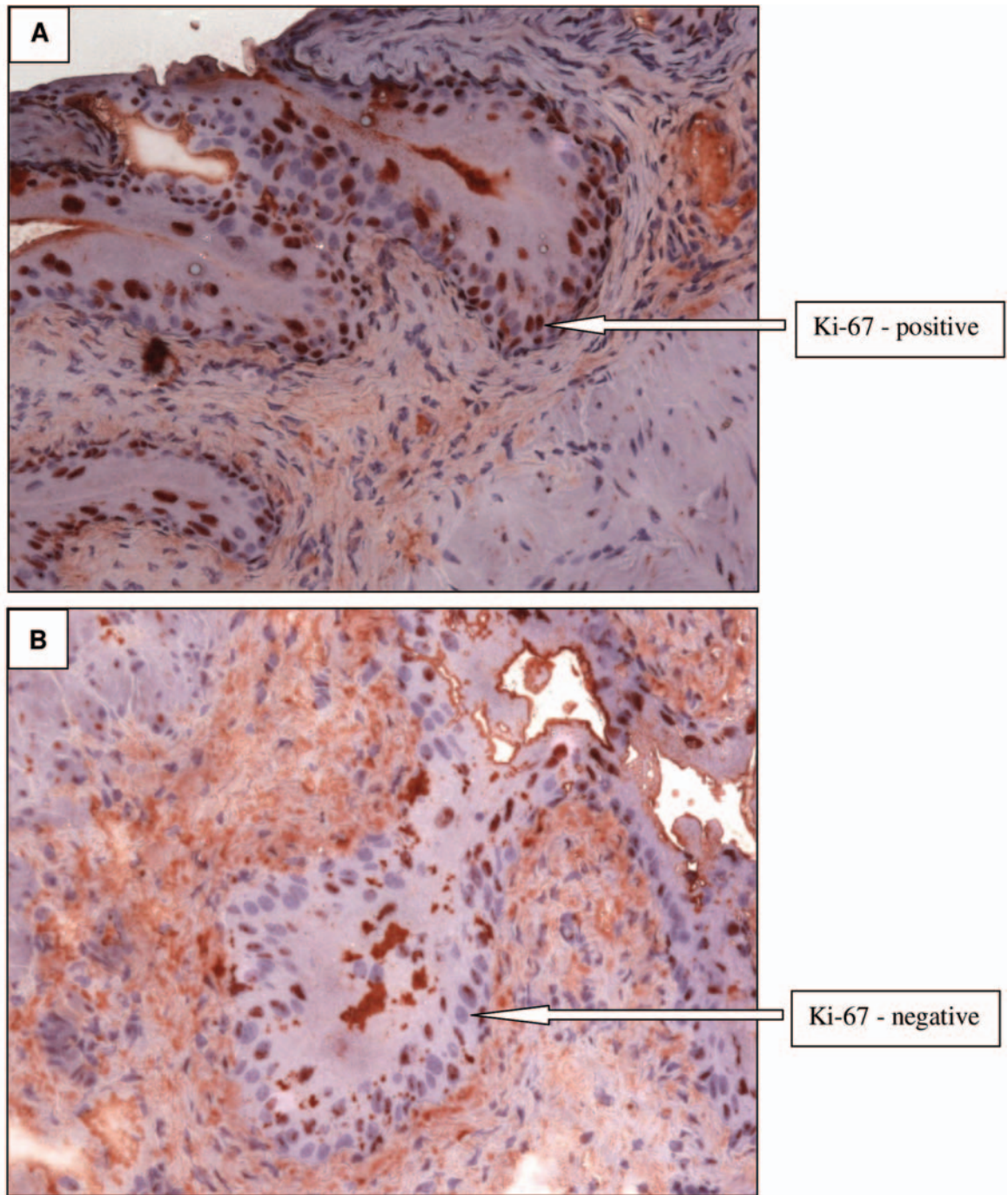
Figure 3. *Immunohistochemical determination of the Ki-67 antigen after intravesical Ki-67 AS-ON application in tumor-bearing C57/Bl6 mice. A, Instillation of NaCl 0.9%; mean 58% positive. B, Instillation of 50 μg Ki-67 FITC-labeled AS-ON mean 43% positive. Representative sections are shown; original magnification, x20.*

animals. Effective uptake is thus virtually impossible (10, 22). To enhance stability several chemical modifications have been proposed. Phosphorothioate oligonucleotides used in this study have a significantly longer half-life *in vivo*. *In vitro*, effective cellular uptake is dependent on transfection reagents such as cationic lipids. Duggan *et al*. have shown in *ex vivo* models with human bladder specimens that excellent intracellular uptake occurs only after 1 hour contact time when given in a liposomal formulation (23).

Low systemic absorption through the bladder wall and direct accessibility of the bladder mucosa make intravesical therapy an excellent route of administration. However, uptake of AS-ON was postulated to be limited by the glycosaminoglycan layer of the inner bladder surface. Additionally, anionic charge and the inherent size of the effector molecules may inhibit the intracellular passage through the cellular lipid bilayer (24). Currently, no systemic studies on humans exist to determine whether carrier systems are required for the efficient uptake of AS-ON into the bladder transitional epithelium.

This study has shown that the Ki-67 directed phosphorothioate AS-ON are able to enter the urothelium and the tumor cells and that no lipid carrier molecule is required. Glackin *et al.* previously demonstrated that uptake of Bcl-2 directed phosphorothioate AS-ON in normal urothelium did not require lipid carrier molecules. The authors consider this finding especially important in the treatment of diffuse urothelial cancer (carcinoma *in situ*), as the targeted therapy with AS-ON applied intravesically has to penetrate the normal urothelium (9).

Here, the availability of the AS-ON 12 hours after intravesical instillation in the cytoplasm was demonstrated. Duggan *et al.* confirmed these investigations as 4 hours after intravesical application of Bcl-2 directed phosphorothioate AS-ON, the AS-ON accumulated in the nuclei and the uptake in the cytosol showed highest intensity after 12 hours (23).

After intracellular accumulation AS-ON are expected to exert their effects by inihibtion of the target gene. A marked inhibition of Ki-67 mRNA and protein expression after Ki-67 AS-ON treatment in cell culture has been previously shown (17). In this study, target evaluation was performed as a supplemental experiment to the uptake studies in a small series. Down-regulation of the target protein was noted immunohistochemically. Furthermore, a significant knock down was also seen in RT-PCR evaluation (data not shown).

Clinical trials using first- or second-generation oligonucleotides have shown that treatments are well tolerated. Initial fever and chills were common side-effects. Additionally, hypotension, liver dysfunction and myelosuppression were reported.

When AS-ON were applied systemically no side-effects were noticed at therapeutical doses in this study. The calculated LD_{50} was >1,000 mg/kg b.w., a much higher dosage compared to any intended treatment dose. A high uptake of AS-ON was demonstrated in the bladder and not in the carcass or plasma in the intravesical approach. The high local availability and low systemic concentration make the bladder an ideal organ for local cancer treatment.

One single instillation of chemotherapeutic agents within 6 hours of TUR is able to reduce the disease recurrence rate by about 50% (25) and is therefore advocated in all non muscle-invasive bladder cancer. The postulated mechanisms of early chemotherapy instillation are destruction of circulating tumor cells that could implant at the site of resection, or other sites, and chemoresection of residual tumor. The reported results justify a therapy with Ki-67 antisense oligonucleotides either as early instillation and/or as repeated instillation thereafter.

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