Atu027, a Liposomal Small Interfering RNA Formulation Targeting Protein Kinase N3, Inhibits Cancer Progression

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Abstract

We have previously described a small interfering RNA (siRNA) delivery system (AtuPLEX) for RNA interference (RNAi) in the vasculature of mice. Here we report preclinical data for Atu027, a siRNA-lipoplex directed against protein kinase N3 (PKN3), currently under development for the treatment of advanced solid cancer. In vitro studies revealed that Atu027-mediated inhibition of PKN3 function in primary endothelial cells impaired tube formation on extracellular matrix and cell migration, but is not essential for proliferation. Systemic administration of Atu027 by repeated bolus injections or infusions in mice, rats, and nonhuman primates results in specific, RNAi-mediated silencing of PKN3 expression. We show the efficacy of Atu027 in orthotopic mouse models for prostate and pancreatic cancers with significant inhibition of tumor growth and lymph node metastasis formation. The tumor vasculature of Atu027-treated animals showed a specific reduction in lymph vessel density but no significant changes in microvascular density. [Cancer Res 2008;68(23):9788–98]

Introduction

RNA interference (RNAi) can be used as novel therapeutic modality through specific silencing of therapeutically relevant genes in vivo (1–5). In particular, chemically synthesized, small interfering RNAs (siRNA) are currently used as a new class of therapeutic molecules, allowing the controlled down-regulation of pathologically relevant gene expression as for oncogenes in cancer (4). However, in analogy to classic antisense molecules the in vivo application of these molecules is not straightforward. The overall negative charge of siRNA molecules (up to 40 negative charges) and the relatively high molecular weight (12,000–14,000 Da) prevent these from functional uptake of these novel therapeutic molecules in vivo. Besides the inefficient uptake and the degradation in endosomal compartments on the cellular level, unformulated siRNAs are rapidly cleared by renal excretion from the blood stream when administered i.v. (5).

To overcome these limitations, delivery technologies for synthetic siRNAs are currently being developed, enabling the use of chemically synthesized siRNA for therapeutic purposes (for a technology review, see refs. 2, 4, 6–8). With respect to cancer, a variety of nonviral nanoparticles (50–200 nm) have been recently reported to be suitable for RNAi-mediated tumor growth inhibition in experimental mouse models (summarized in ref. 6). In most cases, various siRNA carriers such as cyclodextrin (transferrin receptor targeted; ref. 9), PEI (RGD-targeted PEG-PEI; ref. 10), DOTAP (self-assembled LPD-nanoparticle; nanosized immunoliposome; refs. 11, 12), AtuFECT (AtuPLEX; refs. 5, 13), or other cationic lipids (14–17) have been developed for efficient complexation and delivery of siRNAs. The addition of targeting ligands (transferrin, anisamide, RGD-peptide, poly-arginine, anti-TfR single-chain antibody fragment/TfRscFv, DNA) and additional lipid moieties (PEG-lipids, cholesterol, endosomolytic helper lipids, or peptides) or the overall morphology of the generated nanoparticles (characterized by charge and particle size) supposedly confer targeting specificity to either cancer cells and/or tumor vasculature. Likewise, siRNAs targeting the expression of oncogenes (EphA2, EWS-Fli-1, Raf-kinase, EGFR/Her2) or angiogenesis-promoting genes (VEGFR2 and CD31) were chosen for RNAi-mediated therapeutic intervention in oncology.

It remains debatable by which mechanism siRNA-containing nanoparticles (>60 nm) can escape the circulation and cross the endothelial barrier to efficiently penetrate the tumor tissue. However, in contrast to other organ parenchyma, the vascular bed (endothelial cells) is directly accessible for systemically administered particles and therefore predestinated to endocytosis of siRNA-loaded particles, offering the reasonable approach for RNAi-based therapeutic modulation of the vasculature. Hence, an angiogenic approach by means of "formulated siRNA-inhibitor" targeting the tumor vascular compartment might bear valuable strategic and therapeutic advantages.

We recently described a novel liposomal siRNA formulation based on cationic lipids (siRNA-lipoplex/AtuPLEX), containing neutral fusogenic and PEG-modified lipid components, for improved pharmacokinetic properties, cellular uptake, and efficient siRNA release from the endosomes after endocytosis. Using this formulation, we have shown a selective inhibition of gene expression in endothelial cells in vivo as shown by target-specific knockdown of CD31 [platelet endothelial cell adhesion molecule-1 (PECAM-1)] or TIE-2 in various mouse tissues (5, 18). Moreover, we showed therapeutic efficacy in xenograft mouse tumor models after systemic administration of CD31 targeting siRNA-lipoplexes (13). The observed efficacy was not related to toxic side effects including unspecific stimulation of the innate immune system. Repeated dosing of the AtuPLEX does not elevate systemic levels...
of cytokines such as IFN-α or interleukin (IL)-12 (5, 13). These results laid the foundation for the preclinical development of this novel liposomal siRNA formulation for RNAi therapeutics in oncology.

Here we discuss preclinical data on Atu027, a lipoplexed siRNA molecule specifically targeting the expression of protein kinase N3 (PKN3). PKN3 has previously been identified as a downstream effector of the phosphoinositide-3-kinase (PI3K) signaling pathway (19). Loss of PKN3 function in vascular and lymphatic endothelial cells by RNAi suggested PKN3 as an appropriate molecular target for an antiangiogenesis therapeutic strategy. We show the therapeutic potential of Atu027 for tumor treatment in orthotopic mouse tumor models along with PKN3 gene silencing in vivo. Furthermore, we noticed changes in the tumor lymphatic vasculature on Atu027 treatment, suggesting an anti-lymphangiogenesis mode of action for the inhibition of tumor growth and metastasis.

Materials and Methods

siRNA molecules and preparation of siRNA-lipoplexes. siRNA molecules (AtuRNAi, blunt-ended 23-mer, alternating 2′-O-methyl modified) were previously described (20) and were synthesized by BioSpring. To screen for PKN3 mRNA inhibition, eight different mouse and human specific siRNA molecules were designed by bioinformatics means with the screen for PKN3 mRNA inhibition, eight different mouse and human PKN3-specific siRNA molecules were designed by bioinformatics means with the human/mouse-specific PKN3(3) lead siRNA sequence, sense strand 5′-aguacggaauacuucgas-3′ (NM_013355.3); for the rat studies, a rat PKN3(3)-specific siRNA sequence, sense strand 1′-O-methyl modified) was used as control. Complete sequence homology between the PKN3(3) siRNA sequence and the corresponding cynomolgus PKN3 mRNA was confirmed by cloning and sequencing of the corresponding cynomolgus cDNA derived from two different tissues (data not shown). A list of the siRNA sequences used in this study is shown in Supplementary Table S1. All AtuPLEXes (siRNA-lipoplexes) were prepared as described previously (5). The physicochemical properties of Atu027 particles are summarized in Supplementary Fig. S1.

*In vitro* transfection, immunoblotting, and functional assays. The siRNA-lipoplex containing 0.28 mg/mL siRNA (19 μmol/L) was further diluted to a final concentration of 1 to 20 nmol/L siRNA in 10% serum-containing cell culture medium. Human HeLa, PC-3, and murine EOMA cell lines were obtained and cultivated according to the American Type Culture Collection recommendation. Human umbilical vascular endothelial cells (HUVEC) and human microvascular endothelial lung lymphatic cells (HMVEC-L-Ly) were obtained from Lonza.

The following antibodies were used for immunoblotting: rabbit anti-PTEN (Cell Signaling), rabbit anti-PKN3 (19), mouse anti–Hsp60 (BD Laboratories), rabbit anti–Akt-1 (Cell Signaling), and mouse anti-tubulin (Oncogene). For the Matrigel assay, HUVEC and HMVEC-L-Ly cells were trypsinized 72 h posttransfection, counted, and seeded on Matrigel basement membrane matrix (Becton Dickinson) with a seeding density of 100,000 per 24-well plate. At the same time point, cells were lysed for Western blotting to measure PKN3 protein expression. Photographs were taken after 6 h to evaluate tube formation. In the transmigration (Boyden chamber) assay, HUVEC cells were trypsinized 72 h posttransfection, counted, and seeded onto Fluoroblok inserts in serum-free medium. Bottom wells were filled with serum- and growth factor–containing medium as chemoattractant. At the same time point, cells were lysed for Western blotting to show PKN3 knockdown. After 6 h, migrated cells were visualized by staining with SYTOX Green (Molecular Probes), and photographs were taken to determine cell number. HUVEC cells were seeded for the scratch assay onto 24-well plates at 200,000 per well 48 h after transfection with siRNA-lipoplexes. The next day, confluent monolayers were scratched and photographed. Photographs were taken at 0, 3, and 6 h after scratching. In parallel, whole-cell lysates were generated for subsequent immunoblotting.

**RNA in vivo and tumor xenograft experiments in rodent species.** Eight-week-old male NMRI-nu/nu mice (Harlan) and female nonobese diabetic severe combined immunodeficient mice (Charles River) were used...
in this study. For knockdown experiments, siRNA-lipoplex was administered i.v. by low-pressure, low-volume tail vein injection at 300 µL/30 g mouse of a lipoplex solution containing 0.28 mg/mL siRNA and 2.17 mg/mL lipid (equivalent to a dose of 2.8 mg/kg siRNA and 21.7 mg/kg lipid) for high dose. For dose titration, siRNA-lipoplex was diluted in 270 mmol/L sucrose to keep the administration volume of 300 µL/30 g mouse constant.

For infusion studies, mice and rats catheterized via jugular vein (Charles River) received one daily 4-h i.v. infusion with lipoplexed siRNA solution containing 0.14 mg/mL siRNA and 10.9 mg/mL lipid (equivalent to a dose of 2.8 mg/kg siRNA and 21.7 mg/kg lipid) of 20 mL/kg body weight for the high dose on 4 consecutive days. For dose titration, siRNA-lipoplex solutions were diluted in 270 mmol/L sucrose to keep the administration volume constant.

The orthotopic prostate tumor model was previously described (19, 21). In the orthotopic pancreas tumor model, 1.0 × 10⁶ DanG cells/10 mL PBS were injected into the pancreas parenchyma under total body anesthesia. All animal experiments were done according to approved protocols and in compliance with the guidelines of the Landesamt für Gesundheit und Soziales Berlin, Germany (no. G0077/05).

In vivo RNAi in nonhuman primates. The monkey experiments were done at a certified German contract research organization. The study was conducted in compliance with Good Laboratory Practice regulations according to the “Chemikaliengesetz,” current edition, in Germany and according to the “OECD Principles of Good Laboratory Practice” Document Nos. 1 and 13 ENV/MC/CHEM(98)17, ENV/JM/MONO(2002)9. Animal care was in compliance with the “Tierschutzgesetz” (German Federal Animal Welfare Act) as promulgated on February 17, 1993, published in the “Bundesgesetzblatt” (Federal Law Gazette) part I, No. 7 on February 26, 1993, amended on May 25, 1998, and published in the Federal Law Gazette part I, No. 30, dated May 29, 1998. Male and female Cynomolgus monkeys received eight times either 270 mmol/L sucrose solution or Atu027 diluted in 270 mmol/L sucrose every 4th day by 4-h i.v. infusions. The administration volume was 10 mL/kg body weight/h. Five different Atu027 doses were administered: 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg (dose levels refer to the siRNA). Animals were sacrificed 24 h after last infusion on day 30, and lung tissue samples were analyzed (n ≥ 2 per group) by quantitative reverse transcription-PCR (RT-PCR) (TaqMan) and Quantigene 2.0 assay or by immunoblot for PKN3 mRNA and protein expression.

qRT-PCR (TaqMan). For mRNA quantification, tissues were dissected and instantly snap-frozen in liquid nitrogen. RNA isolation and TaqMan RT-PCR have been described previously (5). The sequence of the primers in TaqMan can be obtained on request.

Histologic analysis and determination of microvessel density and lymphatic vessel density. Mice were sacrificed and tissues instantly fixed in 4.5% buffered formalin for 16 h and processed for paraffin embedding (PC-3 model) or mounted with optimum cutting temperature medium and instantly snap-frozen in liquid nitrogen (DanG model). Paraffin sections (2 µm) or cryosections (10 µm) were cut and placed on glass slides. Tissue sections were stained according to standard protocols with goat anti-CD31/PECAM-1 (Santa Cruz Biotechnology), rat anti-CD34 (Cedarlane Labs), or rabbit anti–LYVE-1 (Angiobio for paraffin sections, Angiobio).
Relatech for cryosections) to visualize blood or lymph endothelial cells in the sections. The number of microvessels was determined by counting CD31/CD34–positive vessels or LYVE-1–positive vessels in tumor sections (22, 23). Sections were evaluated blinded by up to three investigators. Four so-called “vascular hotspots” were identified at low magnification (×25) and the number of vessels was counted at high magnification (×200) with a Zeiss Axioquant light microscope. Vessel number as vascular units was evaluated regardless of shape, branch points, and size lumens (referring to “vessel per field”).

siRNA plasma level profiling. Concentrations of the PKN3-siRNA antisense and sense strand were measured independently in liver-heparin plasma using a modified sandwich hybridization assay (24). The method development for the siRNA quantitation assay will be published in detail elsewhere.4

Statistical analysis. Data are expressed as means ± SEM. Statistical significance of differences was determined by the Mann-Whitney U test. P < 0.05 was considered statistically significant.

Results

Atu027-mediated PKN3 inhibition and in vitro loss-of-function analysis in endothelial cells. PKN3 is a downstream effector of the PI3K/Pten signal transduction pathway (19). Here we set out to analyze the biological/physiologic consequences of PKN3 inhibition in cultured vascular endothelial cells and lymphatic endothelial cells. First, we screened target-specific, blunt-ended, nuclease-stabilized (alternating 2′-O-methyl modified) siRNA molecules (AtuRNAi; ref. 20) targeting PKN3 expression in respective cell lines. For both the in vitro and the subsequent in vivo applications, we used the previously described and characterized siRNA-lipoplex formulations (AtuPLEX; for a detailed description, see ref. 5). Five of eight siRNA molecules (AtuRNAi; see Materials and Methods) showed a marked decrease in PKN3 protein expression 48 h posttransfection (Fig. 1A) in HeLa cells and two vascular endothelial cell lines, HUVEC along with EOMA (mouse hemangioendothelioma endothelial cells). A titration of the lipoplexed siRNA PKN3(3) lead sequence, hereafter named Atu027, revealed an IC50 of ~5 nmol/L (concentration for siRNA) in HeLa, HUVEC, and HMVEC-LLy (Fig. 1B).

HUVEC and HMVEC-LLy were treated with Atu027/siRNA PKN3(3)-lipoplex, another potent siRNA PKN3(3)-lipoplex, and two negative siRNA-lipoplexes [siRNA PKN3(1) and siRNA PKN3(4)] or control siRNA LUC-lipoplex. The cells were seeded on extracellular matrix 48 h posttransfection and analyzed (Fig. 1C and D). RNAi-mediated reduction of PKN3 expression in HUVEC and HMVEC-LLy clearly impaired formation of the typical network-like structures on Matrigel as observed for untreated or siRNA LUC-lipoplex–treated cells, suggesting PKN3 function in migration to be required for the formation of endothelial cell tube–like structures. Furthermore, we analyzed PKN3 participation in cell migration, we examined HUVEC cells by transmigration assay (Boyden Chamber assay) and observed a marked decrease in transmigrated cells with reduced PKN3 expression (Supplementary Fig. S2A). This migration phenotype was confirmed in a scratch assay with HUVEC cells treated with Atu027 (siRNA PKN3(3)-lipoplex; Supplementary Fig. S2B). Cells depleted of PKN3 (Supplementary Fig. S2B) showed impaired migration into scratch wounds as compared with controls. To rule out any inhibition of cell proliferation as a direct consequence of PKN3 loss-of-function phenotype in this assay, we analyzed cell growth on plastic dishes after Atu027/siRNA PKN3(3)-lipoplex treatment. PKN3 silencing did not lead to a significant change in the proliferation status of endothelial HUVEC and HMVEC-LLy as well as cancerous Hela and PC-3 cells (data not shown) when compared with untreated or control siRNA LUC-lipoplex–treated cells (Fig. 2A and B). Interestingly, PKN3 gene silencing shown by Western blot was maintained up to 144 h in rapidly proliferating HUVEC and HMVEC-LLy after a single Atu027 transfection (Fig. 2A and B, right). Taken together, Atu027 [siRNA PKN3(3)-lipoplex] target-specifically inhibits PKN3 gene expression, and PKN3 seems to be dispensable for normal cell proliferation but rather plays a role in cell migration and/or adhesion processes required for endothelial tube formation on extracellular matrix. To shed some light on the molecular mechanism of PKN3 signaling in response to vascular endothelial growth factor (VEGF)-A, we analyzed the phosphorylation status of diverse direct effectors of VEGF-R2 such as PI3K/Akt and phospholipase Cγ/endothelial nitric oxide synthase after VEGF-A stimulation in nontransfected as well as siRNA LUC- and siRNA PKN3(3)-transfected HUVEC. No substantial differences in phosphorylation levels were observed in PKN3-depleted and control HUVEC for these known effectors implicated in VEGF-A–mediated pathologic angiogenic neovascularization (refs. 25, 26; Supplementary Fig. S3), and no phosphorylation target could be identified thus far.

Atu027-mediated RNAi in vivo. Systematic administration of Atu027 resulted in suppression of PKN3 expression in vivo when applied as bolus in mice or after (4 hours) infusion in mice, rats, and nonhuman primates. PKN3 mRNA expression was analyzed in different organs by quantitative RT-PCR (TaqMan) after repeated bolus administration of Atu027, control siRNA-lipoplex, and sucrose solution. The PKN3 mRNA reduction was most robust in lung and liver tissues (Fig. 3A) and moderate in tumor tissues, but not apparent in heart and spleen. Importantly, we have previously shown that systemic administration of siRNA-lipoplexes (AtuPLEX) led preferentially to RNAi in the mouse vasculature (5, 13). The moderate PKN3 mRNA reduction observed for s.c. (DU-145) and orthotopic (PC-3) prostate tumor xenografts might reflect the PKN3 expression in nontargeted tumor cancer cells, which cannot be reached with systemic AtuPLEX administration. In other words, target mRNA reduction can be detected in whole highly vascularized tissue lysates or even if target gene expression is not restricted to endothelial cells, can be shown most significantly in the highly vascularized lung.

Furthermore, we analyzed the gene silencing activity of Atu027 given at different doses in mice and rats by repeated daily i.v. infusions (20 ml/kg body weight in 4 hours). In mice, four repeated daily doses of 1.4 and 0.7 mg/kg Atu027 gave rise to a dose-dependent significant mRNA reduction in the lung, whereas in the liver 1.4 mg/kg showed the highest efficacy. Two low doses, 0.35 and 0.18 mg/kg, did not show PKN3 mRNA reduction in any organ at all (Fig. 3B). We also monitored the siRNA levels in mouse plasma during the 4-h infusion twice (at 30 minutes and 3 h 50 minutes) and also 6 and 20 h postinfusion, using a modified capture probe assay (see Material and Methods; Fig. 3B, bottom). Notably, peak plasma concentrations were ~10 nmol/L during infusion of the effective doses (1.4 and 0.7 mg/kg) and, therefore, higher than the in vitro determined IC50 of ~5 nmol/L.

We performed the same study with rats, using a formulated homologous rat PKN3 targeting siRNA (four nucleotide changes in the 23-mer sequence). In rat, we observed PKN3 mRNA reduction

4 C. Lange et al., in preparation.
in the lung and some modest target gene suppression in the liver at the highest dose (2.8 mg/kg), but not at lower doses as seen before (Supplementary Fig. S4, compare with 3B). Therefore, the required effective dose seems to be higher in rats, even though the pharmacokinetic profile for the siRNA levels in rat plasma seemed to be very similar to the one observed in mouse plasma (Supplementary Fig. S4, bottom).

Finally, we conducted a dose range finding study for assessing RNAi in vivo efficacy in nonhuman primates (Cynomolgus monkeys) as part of an initial 4-week subchronic toxicity study for Atu027. Cynomolgus monkeys received, in the initial study, eight repeated 4-h i.v. infusions of Atu027 at doses of 0.3, 1.0, and 3.0 mg siRNA/kg every 4th day. Additionally, control animals were treated in parallel with a 270 mmol/L sucrose carrier solution. Importantly, in vivo gene silencing of PKN3 was observed when compared with sucrose-treated animals for all three different doses on mRNA and protein levels in monkey lung tissue samples harvested 24 h after the last administration on day 29 (Fig. 3C; PKN3 protein: top right;
protein loading controls, p110α and actin; middle row left: Pkn3 mRNA normalized to ubiquitously expressed p110α mRNA). For determination of the lowest RNAi-active dose, a second study in Cynomolgus was carried out including two additional lower doses at 0.03 and 0.1 mg siRNA/kg. Notably, dose-dependent silencing of PKN3 expression was observed, revealing 0.3 mg/kg siRNA as the minimal active dose in monkey (Fig. 3C, b-DNA).

Bioanalytic examination was done with whole blood from these monkey specimens for recording siRNA plasma concentrations at different time points during infusion on day 1 and day 29 to determine possible changes in siRNA plasma clearance after repeated dosing. The lipoplexed siRNA plasma profiles for the infusion on day 1 and day 29 were similar for all five tested doses, indicating that a repeated dosing is feasible (Fig. 3C, bottom). Interestingly, the siRNA plasma concentrations in monkeys were significantly higher when compared with the plasma siRNA concentrations with similar doses administered to rodents (compare bottom graphs of Fig. 3B and C; Supplementary Fig. S3).

**Tumor growth inhibition and reduction of lymph node metastasis by systemic administration of Atu027.** Having shown RNAi in vivo activity of Atu027 in three different species, we investigated the pharmacologic activity in tumor mouse models. Metastases are responsible for the majority (~90%) of deaths associated with solid tumors (27). Therefore, one major objective in the treatment of solid cancer in humans is the prevention and inhibition of invasive tumor growth and metastasis. To test whether Atu027 has therapeutic potential in the prevention of invasive tumor growth and metastasis, we used an orthotopic prostate cancer (PC-3) model (19, 21). This model has the advantage of analyzing both PC-3 prostate tumor growth and assessment of metastatic spread of cancer cells into adjacent lymph nodes. PC-3 cells were transplanted intraprostatically into nude mice, and animals (n = 8 per group) were treated as indicated with sucrose solution, Atu027, or control siRNA<sup>Luc</sup>-lipoplex after tumor establishment with eight consecutive bolus injections every other day at a given dose (Fig. 4A). Notably, we did not observe significant treatment-related changes in body weight in this experiment (Fig. 4A, top right). Tumor volumes and lymph node metastasis volumes and numbers were determined for one group before treatment (pretreatment control group: day 35) to define tumor progression during the treatment period. Regarding therapeutic efficacy, mean tumor and lymph node metastasis volumes further increased in the sucrose- and siRNA<sup>Luc</sup>-treated control group, whereas Atu027/siRNA<sup>PKN3</sup>-lipoplex treatment restrained further tumor growth and, more importantly, the formation of lymph node metastases (Fig. 4B).

To show target-specific gene silencing in Atu027-treated mice, we determined the PKN3 mRNA level in lung tissue extracts 24 h after last treatment by TaqMan-PCR. Down-regulation of PKN3 mRNA in individual mice was detected in comparison with the control siRNA<sup>Luc</sup>-lipoplex–treated mice (Fig. 4C). However, we were not able to show a direct reduction of PKN3 target mRNA in tumor lysates, most probably due to the cell type heterogeneity within the tissue samples and/or the low expression levels of mouse PKN3 mRNA in these low vascularized prostate tumors (data not shown).

The therapeutic efficacy and pharmacologic activity of Atu027 were further proved in a study on PC-3 tumor inhibition using a different treatment schedule (15 i.v. injections every 4th day beginning treatment at day 7) at lower doses compared with the 2.8 mg/kg siRNA dose used before (Fig. 5A). Repeated dosing every 4th day at 1.4 or 0.7 mg/kg, but not 0.3 mg/kg, siRNA resulted in a significant inhibition of further tumor growth and lymph node metastasis formation (Fig. 5B). At the same time, no signs of toxicity or other adverse effects were observed over the entire treatment period, as indicated by the maintenance of stable bodyweight (Fig. 5A, right).
Furthermore, Atu027 was applied in additional mouse tumor models including experimental lung metastasis models (i.v. injection of Lewis lung cancer cells and murine B-16V melanoma cells) and two different orthotopic pancreatic tumor models, supporting the shown therapeutic efficacy (as summarized in Supplementary Table S2). The broad therapeutic effect in these different models is consistent with the hypothesis that Atu027 mediates PKN3 gene silencing in the mouse vasculature and might interfere directly with angiogenesis, lymphangiogenesis, or metastasis processes.

Atu027 reduces the lymphatic vessel density, but not microvessel density, in two different mouse models. We set out to determine whether PKN3 inhibition affects neoangiogenic processes during tumor progression and metastasis. Our previous studies with fluorescently labeled siRNA molecules indicated that tumor vasculature is the preferred cellular target structure within tumors. Analysis of microvasculature density (MVD) or the formation of lymph vessel structures [lymph vessel density (LVD)] in tumor sections from animals treated with Atu027 was carried out by staining with vessel specific markers such as anti-CD34 or anti-CD31 (vasculature) and LYVE-1 (lymphatics). We used two different orthotopic tumor models, a PC-3 prostate cancer or a DanG pancreatic cancer model, treated with the same daily dose of 2.8 mg/kg Atu027, but with different treatment schedules, depending on the tumor model (Fig. 6A and C). A qualitative and quantitative determination of MVD using a CD34-specific (Fig. 6B) or CD31-specific (Fig. 6D) antibody did not reveal a significant difference between Atu027-treated and sucrose-control animals (Fig. 6B and D, top) in the number of blood vessels. In contrast, analysis of LVD in corresponding sections of respective treatment groups revealed a significant reduction of lymphatic vessels in tumor sections of the Atu027-treated animals (Fig. 6B and D).
Although reduction of MVD was not detectable in these particular tumors, we currently cannot rule out a functional impairment of tumor blood vessels.

**Discussion**

There is an unmet medical need for effective cancer therapies that block the progression of solid tumors and prevent the formation of metastasis. PKN3 has been validated as a therapeutic target in cancer cells downstream of PI3K signaling. For example, induced inhibition of PKN3 in PTEN<sup>+/−</sup> prostate cancer cells (PC-3) led to a reduced number of lymph node metastases in orthotopic prostate cancer models (19). In many human sporadic cancers, the PI3K pathway is chronically activated by loss of the tumor suppressor PTEN (28). In endothelial cells, PI3K can be activated by a wide range of vascular growth factors [e.g., VEGF, angiopoietins, basic fibroblast growth factor (bFGF), platelet-derived growth factor-B, ephrin-B2, and transforming growth factor-β superfamily members] or in conjunction with shear stress leading to activation of neovascularization (including tumor angiogenesis; ref. 29). Moreover, a recent publication suggested also a direct function of PI3K in the control of endothelial cell migration during tumor angiogenesis (30). We therefore rationalized that RNAi-mediated gene silencing of PKN3, as a downstream effector of PI3K, in the vasculature of mice may result in inhibition of PI3K-dependent tumoral hemangiogenesis and/or lymphangiogenesis. To support this hypothesis, we validated the PKN3 loss-of-function phenotype in primary HUVEC (vascular endothelial) and HMVEC-LLy (lymph-endothelial) cell culture systems. PKN3 function seemed to be essential for cell migration and adhesion-dependent formation of network/tube-like structures on Matrigel in both endothelial cell types. However, loss of PKN3 function did not significantly affect HUVEC or HMVEC-LLy cell proliferation. From these data, we reasoned that systemic RNAi-mediated PKN3 inhibition might prevent new vessel/endothelial cells from forming appropriate vessel structures without inhibiting the proliferation capacity, reducing the likelihood of target-related toxic side effects caused by PKN3 inhibition in other cell types.

The liposomal siRNA formulation used here (AtuPLEX) has been designed for systemic administration of siRNAs to treat advanced solid cancers (5, 13). Consistent with our previous studies on AtuPLEX-mediated siRNA<sup>CD31</sup> delivery, here we showed that systemic Atu027 administration resulted in therapeutic efficacy in several tumor mouse models. The therapeutic effect is in particular pronounced in the inhibition of lymph node metastasis (PC-3 model) and invasive growth (see Supplementary Table S2; data not shown). In parallel, we succeeded to show Atu027-mediated RNAi in vivo after systemic administration of siRNA by measuring target mRNA quantity in the surrogate lung tissue from three different species. Because PKN3 is also highly expressed in nontargeted cells of the primary tumor, reduction in PKN3 expression within the tumor endothelial cells cannot be easily detected without enrichment of this particular cellular entity.

Tumor angiogenesis, the new generation or modulation of tumor vessels, has been recognized as a hallmark step during carcinogenesis, promoting tumor metastasis (31–33). In view of this, vascularized human tumors such as non–small-cell lung cancer have been found to metastasize early in carcinogenesis to regional lymph nodes, correlating with the degree of lymphangiogenesis.
Additionally, in humans, tumor-associated lymphangiogenesis correlates with lymph node metastasis and prognosis of several tumor entities (36, 37). Our data on Atu027 implicate a function in controlling lymphatic vessel growth, making an anti-lymphangiogenic cancer therapy conceivable. Lymphangiogenesis is currently being considered as an attractive alternative strategy for preventing metastasis (as shown for targeting NRP2; ref. 38). It is suggestive that our observed reduction in LVD, owing to Atu027 treatment, may be the main reason for the reduced metastatic spread of PC-3 cells into regional lymph nodes (Fig. 4B and 5B). At the same time, MVD seemed to be unchanged after Atu027 treatment, which does not ultimately indicate for a missing contribution of the vasculature on tumor progression. The lack of changes in MVD at the end of treatment might reflect that existing mature blood vessels were not disrupted, but additional neo-angiogenic processes became blocked during treatment leading to smaller tumors. To clarify this issue, tumors needed to be harvested from control and Atu027-treated animals at early and late time points. Interestingly, in contrast to Atu027, systemic treatment of mice with bevacizumab, a VEGF neutralizing monoclonal antibody (for summary, see ref. 39), clearly resulted in blocked tumor vessel growth and, consequently, reduced MVD at the end of treatment in Figure 6.

LVD, but not MVD, seems to be reduced in orthotopic prostate and pancreas cancer tumors as revealed by immunohistologic staining of respective tumor tissue sections from Atu027- (lipoplexed siRNA^PKN3) and sucrose-treated mice. A and C, experimental design and treatment schedules for the PC-3 prostate cancer (A) or the DanG pancreatic cancer (C) model. In addition, mean tumor volumes in the prostate (A) or the pancreas (C) model at end of treatment (day 48 or day 22 after cell transplantation, respectively) are shown. B and D, MVD and LVD assessment for tumor sections from vehicle (sucrose)— or Atu027-treated groups by counting CD31/CD34—positive blood vessels and LYVE-1—positive lymph vessels. The number of blood and lymph microvessels was quantified per field tumor section (right). Columns, mean of 12 to 14 samples per group; bars, SEM. *, P < 0.05, Mann-Whitney. Two representative pictures of stained tumor sections displaying CD31/CD34—positive or LYVE-1—positive vessels in respective tumor entities are shown.
our xenograft models (data not shown). Notably, even induction of excessive angiogenesis after Dll4 inhibition was suggested to block tumor growth, contrasting the classic antiangiogenic concept (i.e., MVDR reduction) for effective anticancer therapy (40). Finally, the vessel normalization rather than the disruption of tumor vasculature has been implied to provide antiangiogenic therapy benefits (41). Additional systemic effects of Atu027 treatment and PKN3 inhibition, such as direct obstruction of circulating and/or disseminated tumor cells as well as endothelial progenitor cells, macrophages, or other bone marrow–derived cells, can be proposed as contributing to the onset of metastasis (42–44). However, we have no direct evidence of RNAi-mediated inhibition of PKN3 in these particular cell types thus far. Finally, it should be noted that liposomal formulation of a different poten PKN3 siRNA (Supplementary Fig. S5A; siRNA PKN3(71)) had a similar antitumor effect, as shown in another prostate cancer xenograft (LNcap; Supplementary Fig. S5), supporting the proposed therapeutic PKN3 loss-of-function effect. A sequence and target-independent TLR-mediated antiangiogenesis effect after local injection (intravitreal) of naked siRNAs has been proposed recently (45). However, we have no evidence for TLR activation with formulated (AtuPLEX), 2′-O-methyl modified, 23-mer, and blunt-ended siRNAs in vitro and after repeated systemic administration in mice (Supplementary Fig. S6e). Beyond the already published data (5, 13) on lack of IL-12p40 and IFN-γ stimulation, we expanded the panel of analyzed cytokines by IL-6, the already published data (5, 13) on lack of IL-12p40 and IFN-γ, and blunt-ended siRNAs.

References

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