Atu027 Prevents Pulmonary Metastasis in Experimental and Spontaneous Mouse Metastasis Models

Ansgar Santel, Manuela Aleku, Nadine Röder, Kristin Möpert, Birgit Durieux, Oliver Janke, Oliver Keil, Jens Endruschat, Sibylle Dames, Christian Lange, Mona Eisermann, Kathrin Löffler, Melanie Fechtner, Gerald Fisch, Christiane Vank, Ute Schaeper, Klaus Giese, and Jörg Kaufmann

Abstract

**Purpose:** Atu027, a novel RNA interference therapeutic, has been shown to inhibit lymph node metastasis in orthotopic prostate cancer mouse models. The aim of this study is to elucidate the pharmacologic activity of Atu027 in inhibiting hematogenous metastasis to the target organ lung in four different preclinical mouse models.

**Experimental Design:** Atu027 compared with vehicle or control small interfering RNA lipoplexes was tested in two experimental lung metastasis models (Lewis lung carcinoma, B16V) and spontaneous metastasis mouse models (MDA-MB-435, MDA-MB-231, mammary fat pad). Different dosing schedules (repeated low volume tail vein injections) were applied to obtain insight into effective Atu027 treatment. Primary tumor growth and lung metastasis were measured, and tissues were analyzed by immunohistochemistry and histology. *In vitro* studies in human umbilical vein endothelial cells were carried out to provide an insight into molecular changes on depletion of PKN3, in support of efficacy results.

**Results:** Intravenous administration of Atu027 prevents pulmonary metastasis. In particular, formation of spontaneous lung metastasis was significantly inhibited in animals with large tumor grafts as well as in mice with resected primary mammary fat pad tumors. In addition, we provide evidence that an increase in VE-cadherin protein levels as a downstream result of PKN3 target gene inhibition may change endothelial function, resulting in reduced colonization and micrometastasis formation.

**Conclusion:** Atu027 can be considered as a potent drug for preventing lung metastasis formation, which might be suitable for preventing hematogenous metastasis in addition to standard cancer therapy.

RNA interference (RNAi) therapeutics have been hailed as a potential new drug modality with the opportunity of addressing novel, nondruggable targets for combating a broad spectrum of diseases such as respiratory infections, metabolic disease, or cancer (1). Recently, a small number of new RNAi-based drugs have been translated into clinical testing. Atu027 poses a novel investigational therapeutic agent, currently being tested in a Phase I clinical trial in oncology (2). This drug is composed of a liposomally formulated small interfering RNA (siRNA) targeting the expression of PKN3 specifically in the vascular endothelium (for details, see refs. 3, 4). This targeting encompasses virtually all vascular beds of different organs including the tumor vasculature. Hence, an antiangiogenic mode of action was proposed resulting in prevention of tumor invasion and metastasis (2).

Despite substantial progress in oncology toward successful treatment of certain operable tumor entities, metastasis is still the primary cause of severe morbidity and mortality in cancer. The occurrence of metastasis in cancer patients is often linked to poor prognosis and remains a challenge for therapeutic intervention. The metastatic process depends on multiple parameters, such as the composition of the tumor microenvironment, the properties of the primary tumor, and the compatibility of the distant organ that influences the metastatic growth (5, 6). Cancer cells must pass through a sequence of different steps to establish macroscopic metastases. These steps include the escape of a cancer cell from the tumor tissue through invasion into adjacent host tissue, penetration through the extracellular matrix/basement membrane, and entry into the systemic bloodstream (intravasation). Following intravasation, additional steps such as distribution via the circulation, arrest in the microvasculature, extravasation into the parenchyma of distant organs, and cell proliferation at these ectopic sites occur resulting ultimately in the formation of secondary colonies (“colonization”). Finally,
The development of RNA interference therapeutics holds great promise in finding new treatment opportunities in oncology. Atu027, a novel RNA interference–based therapeutic modality based on liposomal small interfering RNA targeting PKN3, has recently entered a Phase I clinical trial in patients with advanced solid tumors and exemplifies a successful translational “bench-to-bedside” approach. This study underscores the initial findings on the antimetastatic pharmacologic activity of Atu027, affecting both lymphatic and hematogenous dissemination. Furthermore, these results provide new insights in the mechanisms of action as well as in its pharmacologic potential, which is relevant information for future Phase II clinical trial design and conduct.

Materials and Methods

Preparation of siRNA lipoplexes and siRNA molecules

All siRNA lipoplexes were prepared as described previously by mixing liposomes containing the novel cationic lipid AtuFECT01, the neutral helper lipid DPhyPE, and the PEGylated lipid DSPE-mPEG with siRNA at a final concentration of 0.375 mg/mL siRNA and 2.89 mg/mL total lipid (3, 4). Physicochemical properties of Atu027 particles and PKN3 siRNA sequence are as published (2). The following siRNAs were used as negative controls: luciferase (Luc, unrelated control; ref. 4) and “Pool” (which refers to an equimolar mixture of 30 different control siRNAs). All siRNA molecules were stabilized against nucleases by incorporation of alternating 2′-O-methyl-nucleotides (21).

In vitro transfection and Western blot analysis

Human umbilical vein endothelial cell (HUVEC) cells were purchased from Lonza and cultured as recommended. Transfection and subsequent immunoblotting were carried out as described previously (22). Briefly, HUVEC cells were transfected (~12 hours after cell seeding, and different amounts of Atu027 or control siRNA lipoplex solution diluted in 10% serum–containing medium were added to the cells to achieve transfection concentrations in a range of 1 to 40 nmol/L siRNA. At 24 to 96 hours after transfection, cells were lysed and subjected of lung metastases was significantly decreased as assessed in an experimental lung metastasis model (18).

PKN3, a member of the AGC kinase family [a serine/threonin kinase subfamily of which members share similarities in the catalytic kinase domain to PKA (also known as PKAC), cyclic guanosine 3′,5′-monophosphate–dependent protein kinase, and protein kinase C; ref. 19], has been validated as a promising novel therapeutic target in prostate cancer cells for inhibiting tumor progression and lymph node metastasis formation (20). These initial loss-of-function studies have revealed that PKN3 mediates malignant cell growth downstream of chronically activated phosphoinositide 3-kinase (PI3K) pathway (20). Recently, PKN3 has also been considered as a suitable therapeutic target for modulating tumor-associated angiogenesis, because loss of function analysis with Atu027 in cultured primary endothelial cells revealed an essential role of PKN3 for endothelial tube formation and migration (2). Additional preclinical pharmacologic studies with Atu027 showed its inhibitory effect on invasive tumor growth and regional lymph node metastasis in orthotopic pancreatic and prostate cancer mouse models. Finally, systemic i.v. administration of Atu027 has been shown to suppress PKN3 expression in a RNAi-mediated and dose-dependent manner in rodents and nonhuman primates (2). In this manuscript, we further evaluated the pharmacologic activity of Atu027 on lung metastasis in both experimental and spontaneous pulmonary metastasis models.

Translational Relevance

The development of RNA interference therapeutics holds great promise in finding new treatment opportunities in oncology. Atu027, a novel RNA interference–based therapeutic modality based on liposomal small interfering RNA targeting PKN3, has recently entered a Phase I clinical trial in patients with advanced solid tumors and exemplifies a successful translational “bench-to-bedside” approach. This study underscores the initial findings on the antimetastatic pharmacologic activity of Atu027, affecting both lymphatic and hematogenous dissemination. Furthermore, these results provide new insights in the mechanisms of action as well as in its pharmacologic potential, which is relevant information for future Phase II clinical trial design and conduct.

metastases become established after outgrowth of micrometastases into angiogenic macroscopic tumors at the secondary organ sites (7). Novel cancer therapeutics could aim at all these different steps during metastasis as to interfere with cancer cell dissemination and even more importantly with the progressive outgrowth of macroscopic metastases. This can be achieved by targeting the metastatic behavior of the disseminating cancer cell itself but also by modulating the host’s environment, such as the circulatory system, the immune system or the quality of tissue structures at the secondary metastatic site (“metastatic niche”; ref. 8). In this context, changes in vascular permeability, hemostasis, as well as the premetastatic niche formation contribute profoundly to the colonization and outgrowth of metastases (5, 8, 9). For example, maintenance of vascular barrier function is important for controlled paracellular transport between the bloodstream and the tissue parenchyma. Therefore, changes in the barrier function have a direct implication for tumor cell entry into the bloodstream as well as during extravasation into secondary sites during hematogenous metastasis. Alterations in the vascular permeability through destabilization of intercellular adhesion within an endothelial monolayer have been suggested to stimulate inflammatory processes and metastasis (10, 11). The vascular endothelial growth factor-A (VEGF-A) protein (also known as vascular permeability factor; refs. 12, 13) is a prominent cytokine, which promotes endothelial cell proliferation during angiogenesis and increases also in vascular permeability (14). VEGF was found to enhance junctional permeability by VE-cadherin (a main adherens junctions protein) internalization through Src kinase (Src) activation associated with the disassembly of endothelial adherens junctions in cultured cells (15–17). Interestingly, Src as a downstream effector of VEGF signaling was shown to affect directly vascular permeability and metastasis in vivo. In Src-KO mice, permeability of the vasculature was reduced and the number
to immunoblotting. The following monoclonal or polyclonal antibodies were used for Western blot analysis: anti-PTEN, anti-VEGF receptor-2 (VEGFR-2), anti-AKT, anti-β-catenin, anti-MEK1/2 (all Cell Signaling), anti-PKN3 (20), anti-FAK (Upstate), anti-Tie2 (Santa Cruz), anti-Redd1 (23), and anti-IAK1 (Becton Dickinson). Immunofluorescence analysis with HUVEC was done as reported previously (22).

Tumor syngraft and xenograft studies in mice

Experimental lung metastasis models. B16V or Lewis lung carcinoma (LLC) cells (1 × 10^6) resuspended in 200 μL PBS were injected into the tail vein of 8-week-old BDF1 mice (Harlan). siRNA lipoplex treatment started 1 day after tumor cell challenge. After necropsy, metastasis to the lungs was evaluated qualitatively by macroscopic photographs and quantitatively by determining lung weight.

Spontaneous lung metastasis models. MDA-MB-435 or MDA-MB-231 cells (5 × 10^6) resuspended in 40 or 50 μL PBS were injected into the mammary fat pad (m.f.p.) of 8-week-old female scid/bg mice (Taconic). Treatment with siRNA lipoplexes started after tumors became established. Tumor volume was determined by caliper measurement and calculated according to the formula length × width^2/2. At necropsy, metastasis burden refers to relative increase in total lung weight. Alternatively, tumors were resected 35 days after orthotopic transplantation of MDA-MB-435 cells. Mice were randomized into four groups according to transplantation tumor volume and subsequently treated with siRNA lipoplexes. Atu027 and negative control siRNA lipoplexes were given i.v. by low-pressure, low-volume tail vein injection at 300 μL/30 g mouse of siRNA lipoplex solution containing 0.28 mg/mL siRNA and 2.17 mg/mL lipid diluted in 270 mmol/L sucrose (equivalent to a dose of 2.8 mg/kg siRNA and 21.7 mg/kg lipid). All animal experiments were done according to approved protocols and in compliance with the guidelines of Landesamt für Gesundheit und Soziales Berlin.

Histology, immunohistochemistry, and microscopy

Mice were sacrificed, and tissue samples of tumor and lungs were instantly fixed in 4.5% buffered formalin for 16 hours and processed for paraffin embedding. Paraffin sections (3 μm) from different areas of each tumor sample were generated and mounted onto glass slides for histologic assessment. For chromogenic staining, tissue sections were processed for immunohistochemistry according to standard protocols with purified antihuman vimentin monoclonal antibody (Santa Cruz), mouse monoclonal anti-Ki-67 (DAKO), and rat anti-CD34 (Cedarlane Labs). Antibody binding was visualized using NovaRED Substrate (Vector Laboratories). Nuclei were counterstained with hematoxylin (Chroma). Sections were examined with a Zeiss Axioscope microscope. The number of microvessels was determined as previously described (2). For confocal analysis of tissue sections, paraffin-embedded tissue sections were (double-)stained by immunofluorescence according to standard protocols (3, 24) with a rat mono-clonal anti-CD34, rabbit polyclonal anti-VE-cadherin (Cayman), or rabbit polyclonal anti-smooth muscle actin (Sigma). Antibody binding was visualized using Alexa dye–labeled secondary antibodies (Invitrogen). Nuclei were counterstained with SYTOX green dye (Invitrogen) or TO-PRO3. Microscopic analysis of microvasculature was done with a Zeiss LSM510 Meta confocal microscope and double/triple staining recorded using the MultiTrack mode. The Zeiss LSM 510 Ver. 3.0 software was used for two-dimensional color-coded height projection of fluorescence intensity maxima.

Quantification of micrometastasis in the lung

Tissue sections stained with antihuman vimentin monoclonal antibody were examined with a Zeiss Axiosplan light microscope. Three areas with high density of metastatic colonies instead of large uncountable metastatic lesions from each lung section were identified and micrometastases (defined as vimentin-positive foci) were analyzed. ImageJ software (Ver. 1.42q NIH) was used to quantify metastasis in lung sections. For ImageJ software–based quantification of micrometastatic density, images were first taken at 20× magnification by light microscopy. The micrometastatic burden was expressed as number of metastasis present in the defined area of 700.43 μm × 554.96 μm.

Immunoblotting and quantitative reverse transcription-PCR (TaqMan) analysis of whole-lung tissue lysates from mice

Analysis of protein and mRNA expression was carried out as described previously (3, 24). The sequence of the primers in TaqMan can be obtained on request.

Statistics

Data are presented as means ± SEM as indicated for each graph. Statistical significance of differences was determined by the Mann-Whitney U test.

Results

Atu027, a liposomal siRNA formulation targeting PKN3 expression in the lung vascular endothelium, prevents the formation of experimental lung metastasis

Atu027 suppresses PKN3 mRNA levels by RNAi in vitro and in vivo, exhibiting an IC_{50} of 5 to 10 nmol/L (in vitro) as determined in various cell culture systems (2). In addition, substantial knockdown of PKN3 in the vascular endothelium of the lungs in rodents and nonhuman primates has been previously reported for Atu027, when administered systemically (bolus or infusion; ref. 2). As summarized previously, different minimal effective doses were observed after 4 hours of continuous i.v. infusions for three different species (mouse, rat, and cynomolgus monkey), and these effective doses for obtaining a PKN3 mRNA knockdown have been correlated in all three species with a C_{max} in plasma of above 5 nmol/L (mouse).
and 10 nmol/L (rat, cynomolgus), respectively (2). Interestingly, a comparable minimal efficacious dose (0.7 mg/kg) for pharmacologic inhibition of tumor progression and lymph node metastasis (orthotopic PC-3 model) has been established previously in appropriate prostate cancer mouse models (2). These and other pharmacology studies formed the basis for the ongoing Phase I clinical trial for Atu027 (http://clinicaltrials.gov/ct2/show/NCT00938574?term=atu027&rank=1).

Because pulmonary vascular endothelium exhibited robust knockdown of PKN3 after Atu027 treatment, we studied its effect on pulmonary metastasis in two experimental lung metastasis models. Tumor cells such as LLC cell line and the melanoma-derived B16V were given by
tail vein injections to enable lung metastasis during a 2-week time period (Fig. 1). This route of transplantation introducing the cancer cells directly into the lung microvasculature circumvents the initial steps of metastasis, in particular the intravasation of disseminating tumor cells. Because both models lead very rapidly to moribund-recipient mice, we immediately treated the animals with Atu027 after tumor cell challenge (for schedules, see Fig. 1A and D). In comparison to the control group treated with the vehicle (270 mmol/L sucrose), tumor growth in the lung was significantly inhibited in the Atu027-treated mice in both models as indicated by the macroscopic pictures (Figs. 1C and F) and by the lung weight measurement relative to the body weight (Fig. 1B and E; a normal mouse lung represents ca. 0.6% of body weight; data not shown). These results suggest that Atu027 treatment reduced the establishment of hematogenous lung metastases.

**Inhibition of pulmonary metastasis after adjuvant therapy with Atu027**

Adjuvant therapy refers to additional supportive treatment after primary cancer care, such as tumor surgery or radiation, suppressing the risk of cancer recurrence. We used the MDA-MB-435 xenograft–based, spontaneous lung metastasis model to evaluate the therapeutic efficacy of Atu027 on metastasis after primary tumor resection. This xenograft was chosen for assessing the general effects of Atu027 treatment on tumor cell dissemination from the primary tumor site (here m.f.p.) via the hematogenous route to the lung as a remote target organ. In contrast to the above-discussed experimental model, these spontaneous lung models resemble more authentically the multiple steps during metastasis, so that critical parameters such as relevant host-tumor interaction, organ-specific outgrowth, and drug exposure inflicting metastasis can be addressed.

To mirror the clinical situation of an adjuvant therapy approach more closely, treatments with Atu027 or vehicle (sucrose) and two different control siRNA lipoplexes (for details, see Materials and Methods: siRNA Pool and siRNA Control) began on d5 after resection of the established primary tumors (d35 post implantation) at the m.f.p. site (Fig. 2A). Treatments twice weekly continued over a 5-week period as indicated in Fig. 2A. Before resection, the mean tumor volume of all cohorts had reached 600 mm$^3$ on day 35 (data not shown). By contrast on day 72, the total tumor burden in the lung, measured by the relative lung weight, was significantly reduced in the
Atu027 treatment group when compared with the two control siRNA lipoplexes and the vehicle-treated groups (Fig. 2B). In addition, inhibition of lung metastasis was verified by histology/immunohistochemistry, visualizing proliferating metastasized human tumor cells through anti-Ki-67 staining (Fig. 2C) compared with control. The analysis of the stained sections revealed a strong reduction of the metastatic load to various areas of the lungs in Atu027-treated mice (Fig. 2C). These results underscore the initial observation in the experimental lung metastasis models (Fig. 1) and show that Atu027 treatment selectively inhibits pulmonary metastasis also in a more clinically relevant model for spontaneous lung metastasis.

**Atu027 suppresses spontaneous lung metastasis but not primary tumor growth in m.f.p. xenograft models**

Atu027 inhibits hematogenous metastasis to the lung without affecting primary tumor growth. Therapeutic efficacy of Atu027 was evaluated in a xenograft mouse model for spontaneous lung metastasis using two highly metastatic cell lines [MDA-MB-435 (Figs. 2 and 3) and MDA-MB-231 (Supplementary Fig. S1)] at the m.f.p. (25, 26).

To show the pharmacologic effect of Atu027 on spontaneous lung metastasis, we established primary MDA-MB-435 tumors in the m.f.p. of immune compromised mice and treated the animals (n = 18/19) with Atu027 or sucrose, over a 45-day period by i.v. bolus injection (twice weekly), starting on day 12 post cell implantation. Tumor growth was monitored during the treatment period, and pulmonary metastasis was analyzed at different time points post treatment by determining lung weights as indirect measure for overall lung tumor burden (see experimental schedule in Fig. 3A). Interestingly, the tumor growth at the implantation site was not dramatically reduced through repeated Atu027 treatment when compared with the sucrose vehicle control group (Fig. 3B; for MDA-MB-231 model in Supplementary Fig. S1B).

In contrast, the formation of spontaneous pulmonary metastasis differed significantly between sucrose (vehicle) and Atu027-treated animals, when comparing lung weight at the two later time points after treatment (Fig. 3C). This observation was confirmed by immunohistochemical analysis of lung sections using the human cancer cell proliferation marker Ki-67. In control animals, the area of necropsy of 6 mice per group at day 0: 58, 68, 76. MDA-MB-435 tumor cell injection m.f.p. 14x i.v. bolus injections twice weekly (2.8 mg/kg siRNA; 21.7 mg/kg total lipid) N = 18-19 SCID/bg mice/group.

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**Fig. 3.** Atu027 inhibits pulmonary metastasis. A, experimental setup of the spontaneous MDA-MB-435 metastasis model. B, m.f.p. tumor growth curve for vehicle/sucrose (circle) and Atu027 (triangle). Group mean volumes of tumors with SEM were plotted over time. The treatment period is highlighted in gray. C, diagrams showing mean lung weight as percentage of body weight for the vehicle (white column) and Atu027 (black column) at indicated time points after tumor cell implantation. Representative pictures of immunohistochemically stained lung lobe sections from tissue samples of corresponding treatment groups. Tumor tissue in the lung was visualized by staining with anti-human-Ki-67 antibody. P values were calculated according to Mann-Whitney test.
proliferative metastatic lesions increased massively over time, invading almost the entire area of the inspected lung lobes at the end point of the experiment (day 76: 76 days post implantation; 19 days post treatment). In contrast, in sections from Atu027-treated mice, only smaller metastatic lesions were scattered across the entire lung parenchyma (Fig. 3C).

**Atu027 impairs establishment of pulmonary micrometastases**

Next, we studied the quality and appearance of metastases more closely at three different time points after end of treatment (days 58, 68, and 76) by visualizing vimentin-positive tumor cells by immunohistochemistry. The overall number of detectable individual tumor cell colonies in Atu027-treated animals was lower than in the sucrose control group (Fig. 4A). This observation has led us to speculate that Atu027 may affect early colony formation of metastatic tumor cells in the lung. As shown in Fig. 3C, no significant difference in lung weight as an indirect measure for metastasis was found at the time point (day 58) immediately after the end of the treatment. However, quantification of individual metastatic clusters defined as all small immunohistochemistry-detectable vimentin-positive cancer cell foci in a size range from 50 to 2,000 μm² (see Materials and Methods; Fig. 4C) showed the sharp reduction in micrometastasis formation after Atu027 treatment at the first time point (day 58; Fig. 4B). This result was also revealed when looking at distinct micrometastatic clusters, comprising individual tumor cells and small clusters ranging in size from 10 to 100 cells instead of outgrown metastatic lesions (Fig. 4C, left: category 1-4). For all three analyzed categories, formation of early micrometastases was already decreased in analyzed sections from Atu027-treated mice in contrast to control at earliest time point (Fig. 4C). Again, from these data, we conclude that Atu027 treatment inhibits profoundly cancer cell dissemination, resulting in a smaller number of early-formed metastatic colonies. In summary, these observations advocate for a pharmacologic effect on both, colonization (dissemination and extravasation) of metastasizing tumor cells at the secondary sites, which is likely to affect consequent metastatic outgrowth.

In support of these results, we also observed a similar effect on micrometastasis and macrometastasis formation when using the slower metastasizing human breast cancer cell line MDA-MB-231 in the m.f.p. xenograft model (Supplementary Fig. S1A). Again, tumor size at the primary site did not significantly differ between the treatment groups (Supplementary Fig. S1B), but the overall metastatic tumor burden in the target organ lung was significantly decreased in the Atu027 cohort compared with vehicle control group, indicating that the antimetastatic effect of Atu027 is not dependent on a particular cell line but seems to act on the lung as a secondary site for metastasis (Supplementary Fig. S1C). Furthermore, Atu027-treated mice exhibited less colonized micrometastases, suggesting again a therapeutic effect on metastatic colonization.

Taken together, these data revealed a pharmacologic effect of Atu027 on pulmonary metastasis inhibition in two independent models rather than on tumor progression at the primary site (m.f.p.).

**Assessment of different treatment schedules for Atu027**

As discussed above, adjuvant treatment with Atu027 adopts an option in preventing metastasis to remote secondary organ sites. In support for future clinical trial designs with regards to treatment regimens (number of doses and duration), we set out to compare four different treatment schedules with Atu027 in the above-described spontaneous MDA-MB-435 lung metastasis mouse xenograft model. We compared the therapeutic effect of eight doses given either once (regimen R1) or twice weekly (regimen R2) over an 8- or 4-week period, respectively. Another regimen (R4) included 16 doses over a treatment period of 8 weeks (twice weekly) or for comparison over a 12-week period with two 4-week treatments separated by a 4-week intermission (R3: 8 + 8 doses; see schematic in Fig. 5A).

As shown in Fig. 5B, the comparison of these different regimens in this mouse model shows an improved therapeutic effect on metastasis with an expanded dosing (8 versus 16 treatments: R2, R4). Interestingly, the group treated continuously for 8 weeks (R4) showed the same inhibitory effect when compared with a group treated in two 4-week cycles with one intermission (R3). These data suggest that a more regimen with separated treatment cycles, resembling rather a schedule used in chemotherapy, may be one option, but a continuous adjuvant treatment might also be reasonable for increasing the therapeutic benefit. However, it should be recognized that the mouse model still gives only limited insight into pharmacologic relevance of these different schedules, so that it remains difficult to extrapolate to clinically relevant treatment regimens in human patients.

**Depletion of PKN3 resulted in elevated levels of VE-cadherin: Implications for Atu027-mediated inhibitory effect on metastasis**

The *in vivo* observations made in the lung metastasis models prompted us to elucidate the potential molecular mechanisms, underlying the prevention of spontaneous lung metastasis on Atu027 treatment. We first analyzed possible antiangiogenic effects of Atu027 with respect to microvascular density (MVD) and endothelial cell remodeling of the vasculature (namely vessel maturation/pericyte coverage) in respective m.f.p. tumors. Histologic assessment with respect to MVD as well as the quality of tumor vasculature (pericyte coverage) was carried out on sections from the primary breast tumors (MDA-MB-231 and MDA-MBA-435; see experiments discussed above). Several tumor tissue sections from Atu027-treated mice and respective control sections (vehicle or control siRNA lipoplex treated) were analyzed, which did not reveal any striking differences in MVD and tumor vessel quality.
thus far (examples given in Supplementary Fig. S2). This observation is consistent with a lack in antitumor efficacy.

In a first attempt of deciphering molecular effects of Atu027 on the cellular level, we did in vitro studies in HUVEC, studying PKN3 loss of function and subsequent possible molecular downstream events. HUVEC cells were transfected with Atu027 to generate PKN3 loss-of-function effects and analyzed for changes in protein levels of selected signaling mediators (receptor and nonreceptor tyrosine kinases) as well as some structural proteins. Western blot analysis of protein extracts from HUVEC depleted of PKN3 revealed elevated levels of VE-cadherin compared with control samples (untreated, siRNA<sup>Luc</sup> lipoplex-treated cells; Supplementary Fig. S3). An increase in VE-cadherin protein levels correlated in a dose-dependent fashion with knockdown efficacy as shown by titration of different Atu027 concentrations (Fig. 6A). Of note, the loss of PKN3 did not affect VE-cadherin mRNA levels, as revealed by TaqMan PCR analysis in HUVECs transfected with Atu027, suggesting that transcription of VE-cadherin was not affected (Fig. 6B). Moreover, loss of PKN3 did not change levels of all other analyzed proteins, such as Src, FAK, β-catenin, MEK1/2, AKT, JAK1, VEGFR-2, and PTEN (Supplementary Fig. S3). Interestingly, some other markers, including the receptor tyrosine kinases, Tie2 and VEGFR-2, did show an increase in expression levels with increased hypoxic condition as a result of increased cell density (Supplementary Fig. S3; e.g., see also protein levels of the hypoxia-responsive Redd1 protein; ref. 23). Additionally, we analyzed VE-cadherin levels in transfected confluent HUVEC by confocal microscopy to confirm the observed correlation. Immunofluorescence staining with anti-VE-cadherin antibody showed VE-cadherin membrane accumulation with increasing intensity (by means of plotting

Fig. 4. Atu027 impairs micrometastasis formation. A, histochemical detection of micrometastasis development over time in lung sections from indicated treatment groups of the MDA-MB-435 metastasis model (see Fig. 3A). B, the diagram summarizes the quantification of vimentin-positive micrometastatic colonies in lung sections from two time points after treatment. Individual micrometastatic colonies were counted in three fields per section (n = 3). Group mean value of counts. C, quantification of separate micrometastatic colony categories (left immunohistochemical panels) as indicated. Results of quantification for each category (right diagrams; white columns, vehicle control; black columns, Atu027). P values were calculated according to Mann-Whitney test.
color-coded fluorescence intensity data: blue, low intensity; red, high intensity) in an Atu027 concentration-dependent manner (Fig. 6C). Elevated anti-VE-cadherin staining at cell-cell adhesion is reflected by an increase in red-colored membrane areas.

Furthermore, we aimed to confirm these findings in vivo. Mice treated i.v. with Atu027 exhibited a decrease in the pulmonary expression of PKN3 on mRNA and protein levels (Supplementary Figs. S4A and B; ref. 2). As discussed above for the in vitro situation, depletion of PKN3 mRNA in the pulmonary vasculature had no effect on VE-cadherin mRNA levels (Supplementary Fig. S4C). However, elevated expression of VE-cadherin in pulmonary blood vessels was identified in lung sections from Atu027-treated mice (Fig. 6D). It should be noted that the data represent merely qualitative rather quantitative assessment and that VE-cadherin upregulation was not evident in all analyzed sections. Unfortunately, the anti-VE-cadherin antibody did not allow assaying for VE-cadherin levels in protein extracts derived from mouse lungs. Recently, enhanced expression of VE-cadherin, the main junctional protein of adherence junctions in endothelial cells (27), was reported as a marker for tumor vessel normalization (28). Preliminary attempts of showing enhanced VE-cadherin protein expression in the tumor vasculature of Atu027-treated mice did not reveal a robust VE-cadherin staining (data not shown).

Nonetheless, it remains conceivable that loss-of-PKN3 function in endothelial cells might result in increased levels of VE-cadherin, suggesting consequences for endothelial adherens junction integrity in the control of vascular permeability or outside-in signaling properties for diverse vascular beds in Atu027-treated animals.

**Discussion**

In this study, we aimed to give a deeper insight into the pharmacologic activity and the possible underlying molecular mechanisms of Atu027, a novel investigational drug currently in Phase I clinical trial for oncology. We propose that Atu027, as a novel liposomal RNAi therapeutic, modulates the properties of the vascular endothelium, which...
ultimately leads to the prevention of hematogenous metastasis. The data we provided suggest a model whereby Atu027-mediated reduction of endothelial PKN3 expression might lead to elevated VE-cadherin levels in the vasculature of the lung as secondary organ site and possibly tumor, too. Perhaps, this change in VE-cadherin levels reduces vascular leakage and the intravasation and extravasation of metastatic tumor cells, resulting in the prevention of metastasis. Metastatic tumor cells need at first to overcome the vascular endothelium of blood vessels and their underlying basal membrane at the tumor site for entering the circulation (intravasation). To establish second site metastatic tumors, the spread cancer cells need to successfully invade (extravasate) from the circulation by again crossing the endothelial barrier into the adjacent parenchyma of a given organ (e.g., lung or liver). Finally, metastases initiate neo-vascularization (angiogenesis; refs. 29, 30). Therefore, systemic action of Atu027 on diverse vascular beds can affect both entry and exit steps of metastatic cancer cells. Nevertheless, we cannot definitively discriminate whether the tumor cell dissemination is blocked at the stage of either intravasation or extravasation. However, the results from the applied two different metastasis models (experimental versus spontaneous) may shed some light on this aspect.

Fig. 6. Atu027 alters VE-cadherin levels. A, concentration-dependent effect of Atu027 on VE-cadherin, as revealed by transiently transfecting HUVEC with Atu027 and control siRNA3 luc lipoplex (Luc, luciferase). Corresponding protein extracts were probed in a Western blot experiment for PKN3 and VE-cadherin; PTEN was used as loading control. Tested siRNA concentrations are indicated: ut, untreated cell extracts. B, Atu027-transfected HUVEC showed concentration-dependent PKN3-mRNA knockdown but no changes in VE-cadherin mRNA levels as measured by TaqMan real-time PCR. Relative amount of mRNA levels of the respective target normalized to PTEN-mRNA are shown for indicated siRNA concentrations (gray columns, siRNA3 luc lipoplex; black columns, Atu027) transfected in HUVEC. C, HUVECs transfected with Atu027 or siRNA3 luc lipoplex (Luc, luciferase) were grown to confluency on coated slides, stained by immunofluorescence with anti-VE-cadherin, and subsequently analyzed by confocal microscopy. Representative pictures are shown, indicating different VE-cadherin levels by the degree of red membrane staining at cellular adhesion sites. D, two examples for pulmonary venous vessels with increased VE-cadherin endothelial staining (arrows) are shown compared with control.
implying Atu027 effects on both metastasis steps. The experimental model mimics already successful intravasation of tumor cells, so that the observed inhibition of metastasis after Atu027 treatment points to impaired extravasation (Fig. 1). On the other hand, the data obtained with the spontaneous model indicate a reduced metastatic colonization (reduced number of micrometastasis) and therefore suggests an impairment of the intravasation step as well (Figs. 2-4; Supplementary Fig. S1). The here applied spontaneous metastasis models bear the following caveat that merely the end point effect of the drug can be evaluated. However, it would be attractive to obtain insights into the early steps of the metastasis process, which would tremendously contribute to the understanding of the mode of action of Atu027 during hematogenous metastasis. More sophisticated imaging methods and mouse metastasis models, for example, could help to track the fate of the cancer cells in real time during all steps of metastasis also with respect to their interaction with the vascular endothelium, as it was shown in recent studies (30, 31). Furthermore, instead of using cultured human cancer cells for orthotopic tumor implantation, so-called surgical orthotopic implantation (32), tumor fragments from surgical cancer specimen from patients can be used for implantation to evaluate the efficacy of a novel drug modality on tumor growth and metastasis in a more "clinically accurate and relevant" model.

The observed lack of antitumor effect of Atu027 on primary tumor growth resembles results from other published tumor inhibition studies with, e.g., MDA-MB-435-derived tumors, suggesting the absence of tumor growth relevant angiogenesis, and support previous findings made in orthotopic pancreatic and prostate cancer xenograft models (2). For example, treatment with the multi-kinase inhibitor E7080 directed against VEGFR-2 and VEGFR-3 or with bevacizumab showed no significant MDA-MB-435 tumor inhibition whereas the lymph vessel dependent MDA-MB-231 m.f.p. mouse model revealed inhibition of tumor growth (33). Furthermore, the pharmacologic effect for Atu027 in the here described study is also reminiscent of Src kinase inactivation. Like in Src-KO mice, metastasis inhibition occurred although tumor growth and MVD remained unchanged between KO and control (wild-type and heterozygotes; ref. 18). Interestingly, a recent report describes a very similar antiangiogenic effect of haploinsufficient expression of the PHD2 gene in the vascular endothelium on metastasis. Syngenic tumors implanted in PHD2-/- mice showed impaired metastasis rather than reduced tumor growth (28). Moreover, on the molecular level, HIF-induced VE-cadherin expression was shown along with improved vessel maturation, whereas MVD was not affected. Again, upregulated VE-cadherin may not only account for restored endothelial normalization and vessel function within the tumor as speculated by Mazzone et al. (28) but also contribute to enhanced cell-cell adhesion and tightening of endothelial monolayer, thereby eventually inhibiting metastasis.

At present, it remains to be clarified whether this anti-metastatic effect of Atu027 is restricted to the lung tissue, wherein strong RNAi-mediated PKN3 suppression occurs in the pulmonary endothelium (2) or whether hematogenous metastasis can also be reduced in other pivotal organ sites of metastasis, such as liver, kidney, brain, and bones. Previous findings with Atu027 applied in orthotopic pancreas and prostate cancer models provided evidence of suppressed local invasion as well as lymph node metastasis (2). The here proposed modulatory effect of Atu027 on the vascular endothelium could also support the initial finding in a way that improved endothelial barrier function virtually results in less tumor-associated swelling/edema development or interstitial fluid pressure and the reduced possibility of lymphatic metastasis. Because the density of tumor vasculature in the here described models were not significantly affected by Atu027 treatment (data not shown), the therapeutic activity of Atu027 stands in sharp contrast to classical antiangiogenic therapeutics such as bevacizumab or sorafenib, both acting on tumor vessel size and number as revealed in preclinical models (34, 35). The difference to these classical antiangiogenic drugs supports the combination of Atu027 not only with conventional chemotherapeutics but also other antiangiogenic agents to potentiate effective cancer treatment (36). The latter aspect is of importance especially with respect to the emerging tendency of tumors to develop resistance against antiangiogenic therapy (37–39).

Our results regarding treatment schedule and frequency in the mouse model system suggest that a prolonged treatment period might have a clinical benefit. However, the frequency of treatment (twice weekly schedule in mice) is difficult to predict for the human system because tumor development in mouse models progresses very rapidly and is likely to require a more frequent treatment, enabling an RNAi-mediated target inhibition in target cell structures with high proliferation rates. The current ongoing Atu027 Phase I study (NCT00938574) does include a repeated treatment period to assess safety issues but the optimal treatment regimen needs to be further evaluated in future Phase II studies.

Taken together, the here presented results with Atu027 in mouse models suggest a therapeutic benefit in preventing metastasis to the lung. This observation has direct implications for future therapeutic applications and proposes that Atu027 when given in an adjuvant setting might be a promising new antimetastatic therapeutic drug. This approach will require a specific selection of patients with potential high metastatic risk, including patients with evidence of inoperable micrometastases or lack of clinically detectable metastasis. This potential adjuvant treatment is supported in particular by our results showing profound inhibition of pulmonary metastasis after resection of the breast xenograft transplantation tumor (Fig. 2), modeling the clinical situation of cancer patients post surgery. Therefore, we conclude from our current preclinical studies that
Au027 represents a new therapeutic modality for inhibition of pulmonary metastasis and may therefore be attractive for further clinical testing in an appropriate Phase II study.

Disclosure of Potential Conflicts of Interest

All authors are employees of and have stock options in Silence Therapeutics AG. O. Keil, A. Santel, and J. Kaufmann are named inventors on patent applications directed to PKN3. O. Keil and J. Kaufmann are named inventors on patent applications directed to Au0PLEX.

References

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Ansgar Santel, Manuela Aleku, Nadine Röder, et al.


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