ABSTRACT
RNA interference (RNAi) is a potent and specific mechanism for regulating gene expression. Harnessing RNAi to silence genes involved in disease holds promise for the development of a new class of therapeutics. Delivery is key to realizing the potential of RNAi, and lipid nanoparticles (LNP) have proved effective in delivery of siRNAs to the liver and to tumors in animals. To examine the activity and safety of LNP-formulated siRNAs in humans, we initiated a trial of ALN-VSP, an LNP formulation of siRNAs targeting VEGF and kinesin spindle protein (KSP), in patients with cancer. Here, we show detection of drug in tumor biopsies, siRNA-mediated mRNA cleavage in the liver, pharmacodynamics suggestive of target downregulation, and antitumor activity, including complete regression of liver metastases in endometrial cancer. In addition, we show that biweekly intravenous administration of ALN-VSP was safe and well tolerated. These data provide proof-of-concept for RNAi therapeutics in humans and form the basis for further development in cancer.

SIGNIFICANCE: The findings in this report show safety, pharmacokinetics, RNAi mechanism of action, and clinical activity with a novel first-in-class LNP-formulated RNAi therapeutic in patients with cancer. The ability to harness RNAi to facilitate specific multitargeting, as well as increase the number of drug-gable targets, has important implications for future drug development in oncology. Cancer Discov; 3(4): 1–12. ©2012 AACR.
INTRODUCTION

RNA interference (RNAi) is an endogenous cellular mechanism for controlling gene expression in which siRNAs bound to RNA-induced silencing complex (RISC) mediate target mRNA cleavage and degradation through a catalytic process involving the Argonaute 2 endonuclease (1, 2). The use of siRNAs to specifically silence genes involved in disease pathogenesis holds promise for the development of a new and far-reaching class of therapeutics (3, 4). Because the RNAi pathway is present in all mammalian cell types, the primary challenge for effective gene silencing in vivo is delivery of the siRNA to the appropriate organ(s) with productive cellular uptake leading to engagement of RISC in the cytosolic. Systemic delivery is required for the widest application of RNAi therapeutics, and to that end much effort has been focused on the development of siRNA formulations that confer “drug-like” properties favorable to delivery and uptake following parenteral administration (5).

Various nanoparticle formulations have been evaluated for their ability to silence targets in vivo (6–10); among these, lipid nanoparticles (LNP) have been shown to be highly effective in delivering siRNAs to the liver and silencing a number of different hepatocyte gene targets across multiple species, including rodents and nonhuman primates (11–14). Delivery to tumors has also been shown in murine orthotopic liver tumor and subcutaneous tumor models, in which both mRNA silencing and on-target pharmacology were observed following i.v. dosing (15). Although results in animal models with LNPs are promising, it remains to be shown whether these findings will translate into an effective way to deliver siRNAs for the treatment of human disease. To examine the activity and safety of LNP-formulated siRNAs in humans, we performed a study to assess the pharmacodynamic changes expected with KSP and VEGF inhibition were also observed. Specifically, within 48 hours of single dose of ALN-VSP to mice with established tumors resulted in dose-dependent suppression of both VEGF and KSP mRNA, with up to 50% reduction observed 24 hours after a 4 mg/kg dose. This finding was accompanied by the generation of specific RNAi-mediated VEGF and KSP mRNA cleavage products, as measured using the 5′ Rapid Amplification of cDNA Ends (5′ RACE) assay (refs. 13–20; Supplementary Fig. S1). ALN-VSP has a particle diameter of 80 to 100 nm and is essentially uncharged with a zeta potential of less than 6 mV at pH 7.4. Consistent with other liposomes of a similar size (21, 22), ALN-VSP distributes primarily to liver and spleen following parenteral administration due to the fenestrated endothelium in those organs. Distribution to tumors with leaky microvasculature containing endothelial pores is thought to occur through the enhanced permeability and retention (EPR) mechanism described for liposomes and other nanoparticles (23, 24).

The organs with demonstrable toxicity in preclinical animal toxicology studies with ALN-VSP were primarily liver in rats and spleen in monkeys. A 50% reduction in spleen weight associated with lymphoid atrophy was observed in monkeys treated with 4 doses of 6 mg/kg of ALN-VSP every 2 weeks but not in control animals treated with the same dose of LNP containing an siRNA-targeting luciferase (Supplementary Table S1). These findings were indicative of an on-target siRNA effect rather than a nonspecific effect of the LNP itself, and the lymphopenia reported in patients treated with a small-molecule KSP inhibitor (25, 26) suggested that KSP rather than VEGF inhibition was underlying the splenic changes in monkeys occurring with ALN-VSP.

In an orthotopic liver tumor model using severe combined immunodeficient (SCID)/beige mice implanted with Hep3B human hepatocellular carcinoma cells, i.v. administration of a single dose of ALN-VSP to mice with established tumors resulted in dose-dependent suppression of both VEGF and KSP mRNA, with up to 50% reduction observed 24 hours after a 4 mg/kg dose. This finding was accompanied by the generation of specific RNAi-mediated VEGF and KSP mRNA cleavage products, as measured using the 5′ Rapid Amplification of cDNA Ends (5′ RACE) assay (refs. 13, 14, 27, 28; Supplementary Fig. S2A). In addition to these effects on mRNA expression, the pharmacodynamic changes expected with KSP and VEGF inhibition were also observed. Specifically, within 48 hours following a single dose of ALN-VSP, tumor cells frozen in mitosis with unipolar mitotic spindles (monoasters) were seen throughout liver tumors in ALN-VSP-treated mice but not in control animals (Supplementary Fig. S2B), consistent with KSP inhibition. In mice given repetitive doses of the LNP-formulated VEGF siRNA (LNP-VEGF), both the reduction in tumor hemorrhage and microvascular density were seen to a degree comparable with results in animals treated with the anti-VEGF antibody bevacizumab (Supplementary Fig. S2C). Following these demonstrations of anti-KSP and anti-VEGF pharmacodynamics, the antitumor activity of ALN-VSP administered as repeated doses over 3 weeks was tested in mice bearing established tumors. Animals treated with ALN-VSP had an approximately 50% improvement in median survival relative to control animals (Supplementary Fig. S2D). In addition to having an impact on hepatic tumors, parenterally administered ALN-VSP also has activity against extrahepatic tumors, as shown by the detection of tumor metastases.

RESULTS

ALN-VSP Composition and Preclinical Safety and Activity

ALN-VSP is composed of an LNP containing 2 different siRNAs targeting VEGF-A (hereafter referred to as VEGF) (16) and kinesin spindle protein (KSP, encoded by the KIF11 gene; ref. 17) in a 1:1 molar ratio. Both siRNAs were chemically modified to reduce their immunostimulatory potential (refs. 18–20; Supplementary Fig. S1). ALN-VSP has a particle diameter of 80 to 100 nm and is essentially uncharged with a zeta potential of less than 6 mV at pH 7.4. Consistent with other liposomes of a similar size (21, 22), ALN-VSP distributes primarily to liver and spleen following parenteral administration due to the fenestrated endothelium in those organs. Distribution to tumors with leaky microvasculature containing endothelial pores is thought to occur through the enhanced permeability and retention (EPR) mechanism described for liposomes and other nanoparticles (23, 24).

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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cell monoasters following ALN-VSP treatment in mice with abdominal peritoneal Hep3B tumor implants (Supplementary Fig. S3A and S3B).

Phase I Study Design and Patient Characteristics

The dose-escalation phase of the study used a 3 × 3 design in which patients were enrolled sequentially on 1 of 7 dose levels (0.1–1.5 mg/kg). This was followed by an expansion phase at the maximum-tolerated dose (MTD). ALN-VSP was administered through either a peripheral angiocath or central line as a 15-minute i.v. infusion via a controlled infusion device using an extension set with a 1.2-μm filter every 2 weeks, with a cycle of therapy defined as 2 doses given over 1 month (Supplementary Fig. S4). All patients were pretreated before each dose with dexamethasone, 8 mg orally the night before and dexamethasone, 20 mg i.v., acetaminophen 650 mg orally, diphenhydramine, 50 mg i.v. (or hydroxyzine, 25 mg orally), and either 50 mg of ranitidine or 20 mg of famotidine i.v. 30 minutes before infusion to reduce the risk of infusion-related reactions (IRR) observed with other liposomal products (29). Tumor measurements by computed tomography (CT) scan were conducted after every 2 treatment cycles. Patients whose disease had not progressed by CT scan after 4 cycles of therapy were eligible to continue treatment on an extension study. A total of 41 patients were enrolled between March 2009 and August 2011, including treatment on an extension study. A total of 41 patients were eligible to continue the study after 4 cycles of therapy (Table 1). All patients were pretreated with either chemotherapy or anti-VEGF/VEGF receptor (VEGFR) agents. The majority had both hepatic and extrahepatic disease. A total of 277 doses (average of 6.8 doses/patient; range, 1–50) were administered on both the phase I and extension studies.

Antitumor and Pharmacodynamic Activity

Among 37 patients evaluable for tumor response, 4 of 24 (16.7%) patients treated at 0.7 mg/kg had disease control (stable disease or better after at least 12 doses over 6 months; Table 2). Seven patients receiving ALN-VSP doses ranging from 0.4 to 1.0 mg/kg whose disease did not progress after the first 4 months went on to the extension study (Supplementary Table S2) and were treated for an average of 11.3 months (range, 5–26 months); these included patients with VEGF-overexpressing tumors such as renal cell cancer (RCC), endometrial cancer, pancreatic neuroendocrine tumor (PNET), and angiosarcoma (30–33). A major response [complete response, defined per Response Evaluation Criteria in Solid Tumors (RECIST) as disappearance of all target and nontarget lesions] occurred in a patient with endometrial cancer and multiple hepatic metastases (Fig. 1A) as well as an abdominal lymph node metastasis, whose disease had progressed after prior chemotherapy and experimental therapy with a Hedgehog inhibitor. Tumor regression was observed after the first 2 cycles of ALN-VSP used at 0.7 mg/kg, and by the end of 6 cycles the patient had achieved a partial response with complete regression of the lymph node metastasis and substantial shrinkage of the liver tumors. A complete response was obtained after 40 doses in this patient, who has remained in remission and completed treatment after receiving 50 doses over 26 months. Two patients with RCC and extrahepatic sites of disease including kidney, lung, and lymph nodes that were progressing following prior therapy with VEGF pathway inhibitors had stable disease at all sites for approximately 8 to 12 months, and a patient with PNET and multiple liver metastases continued on the extension study for 18 months (36 doses) with stable disease (Table 2).

Dynamic contrast-enhanced MRI (DCE-MRI) was conducted at baseline and twice during the week after the first dose of ALN-VSP (see Supplementary Methods for details) to determine whether treatment was associated with a reduction in tumor blood flow indicative of an anti-VEGF effect (34). To be evaluable by DCE-MRI, patients

### Table 1. ALN-VSP phase I trial: demographics and dosing

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total study population, N</td>
<td>41</td>
</tr>
<tr>
<td>Dose escalation (0.1–1.5 mg/kg)</td>
<td>30</td>
</tr>
<tr>
<td>Expansion phase at 1.0 and 1.25 mg/kg</td>
<td>11 (6 at 1.0 mg/kg, 5 at 1.25 mg/kg)</td>
</tr>
<tr>
<td>Median age</td>
<td>57 (range, 34–78)</td>
</tr>
<tr>
<td>Male:female</td>
<td>17:24</td>
</tr>
<tr>
<td>ECOG performance status 0/1, %</td>
<td>44 / 56</td>
</tr>
<tr>
<td>Average number of prior regimens for</td>
<td>massage disease</td>
</tr>
<tr>
<td>metastatic disease</td>
<td>4.3 (range, 0–15)</td>
</tr>
<tr>
<td>Prior chemotherapy/anti-VEGF therapy,</td>
<td>88/61</td>
</tr>
<tr>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Liver/extrahepatic metastases, %</td>
<td>98/88</td>
</tr>
<tr>
<td>Tumor types, N</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>24</td>
</tr>
<tr>
<td>Gynecological</td>
<td>9</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>3</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
</tr>
<tr>
<td>Doses administered</td>
<td>277</td>
</tr>
<tr>
<td>Average number of doses/patient</td>
<td>6.8 (range, 1–50)</td>
</tr>
<tr>
<td>Abbreviation: ECOG, Eastern Cooperative Oncology Group.</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Characteristics of patients with disease control

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Dose level, mg/kg</th>
<th>Tumor type</th>
<th>Best response</th>
<th>Number of doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>021</td>
<td>0.70</td>
<td>Endometrial</td>
<td>CR</td>
<td>50</td>
</tr>
<tr>
<td>037</td>
<td>1.00</td>
<td>Renal cell</td>
<td>SD</td>
<td>17</td>
</tr>
<tr>
<td>040</td>
<td>1.00</td>
<td>Pancreatic neuroendocrine</td>
<td>SD</td>
<td>36</td>
</tr>
<tr>
<td>041</td>
<td>1.00</td>
<td>Renal cell</td>
<td>SD</td>
<td>23</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; SD, stable disease.
Clinical Activity of an RNA Interference Therapeutic in Cancer Patients

Figure 1. Clinical activity of ALN-VSP in patients with cancer. A, complete response in endometrial cancer patient 021 with multiple liver metastases. CT images of metastatic tumors (black arrows) obtained before start of ALN-VSP (pretreatment) and following 12 and 40 doses of drug. B, decrease in blood flow ($K_{\text{trans}}$) in liver tumors of patients treated with ALN-VSP. For 24 patients with evaluable DCE-MRI scans, the average peak change in $K_{\text{trans}}$ for one or more liver tumors occurring during the first week after dose #1 is shown for each patient by dose level. C, DCE-MRI images from patient 012 with metastatic PNET treated at 0.7 mg/kg. Top left, Baseline MRI, coronal view ($K_{\text{trans}}$, T1, T2, T3). Top right, DCE-MRI images of tumor blood flow ($K_{\text{trans}}$ change from baseline) for one or more liver tumors occurring during first week after dose #1 in each tumor. Bottom, corresponding DCE-MRI images of tumor blood flow ($K_{\text{trans}}$ and IAUGC) occurring during first week after dose #1 in each tumor. D, decrease in spleen volume in patients treated with ALN-VSP. Spleen volume was measured on abdominal CT conducted for tumor measurements in 25 evaluable patients with both pre- and posttreatment scans.

were required to have at least 1 liver tumor measuring 2 cm or more in diameter. Both transfer constant ($K_{\text{trans}}$) and initial area under the gadolinium concentration (IAUGC) time curve were measured to assess blood flow and capillary permeability. Among 28 patients across all dose levels with evaluable scans, 46% (13 of 28) had a 40% or more peak average reduction in $K_{\text{trans}}$ (Fig. 1B) although there was no evidence of dose-dependence. Changes in $K_{\text{trans}}$ and IAUGC tended to occur in parallel and were often seen within days of the first dose in both large and small tumors (Fig. 1C).

To determine whether ALN-VSP had the capability to cause the changes in spleen size observed in monkeys, serial measurements of spleen volume were undertaken retrospectively in 25 evaluable patients who had abdominal CT scans conducted for tumor measurements. After 2 to 16 doses of ALN-VSP (patients with more doses had remained on study longer due to lack of disease progression), an average reduction of $\sim$37% ± 25% (range, 8 to $-92\%$) was observed in patients dosed at 0.4 to 1.5 mg/kg (Fig. 1D), with no clear dose response but with the largest effect seen after 12 or more doses. These splenic changes were consistent with the preclinical findings in monkeys and therefore suggestive of an anti-KSP effect.

Although no overall correlation was shown between change in $K_{\text{trans}}$ or spleen volume and tumor response, the patient with endometrial cancer who had a complete response had a more than 50% decrease in $K_{\text{trans}}$ and a 90% decrease in spleen size.

RNAi Proof of Mechanism

Voluntary CT-guided core needle tumor biopsies were conducted in 15 patients dosed at 0.4 to 1.5 mg/kg to measure drug levels and look for evidence of RNAi. Biopsies were taken before the first dose of ALN-VSP and then again 2 to 7 days after treatment. Biopsied metastatic tumors were hepatic in 11 patients and extrahepatic in 4 patients. The biopsies were notable for the considerable degree of inter-patient heterogeneity with respect to the amount of viable tumor, necrotic tumor, fibrosis, and/or normal tissue (e.g., liver) present. This degree of heterogeneity, coupled with the different tumor types, sites of metastasis, and days from dosing to biopsy, placed limitations on the ability to compare quantitative assessments such as drug levels between patients.
Nonetheless, among 12 patients who had biopsies evaluable for both VEGF and KSP siRNAs using quantitative reverse transcriptase PCR (qRT-PCR; Table 3; ref. 35), all had detectable VEGF siRNA after treatment (average concentration of 21.3 ± 39.1 ng/g tissue; range, 0.45–142), and 11 of 12 had detectable KSP siRNA (average, 13.6 ± 20.8 ng/g tissue; range, 0.4–73.3; difference between VEGF and KSP siRNA concentrations not significant). Two of these patients had biopsies that were 96% to 100% viable tumor (#025 with sarcoma muscle metastasis and #042 with melanoma liver metastasis), thereby suggesting drug delivery to tumor itself. Because biopsies from the other 10 patients contained varying amounts of viable tumor mixed with fibrosis/necrosis and/or normal tissue (Table 3), the proportion of KSP and/or VEGF siRNA distributed to tumor versus other tissue types within the biopsy could not be determined.

The 5' RACE assays for VEGF and KSP were conducted on biopsies from all 15 patients. The basal level of specific cleavage product, predicted by the siRNA sequence for each target mRNA and determined by evaluating all of the pretreatment biopsy samples as well as multiple banked tumor and normal liver samples from untreated subjects not on the clinical trial, was 0.7% of total sequences. Among the posttreatment biopsies, 2 patients biopsied 48 hours after dosing, whose liver tumor biopsies were composed predominantly of normal liver and had little to no viable tumor, had a substantial increase in the specific cleavage product for VEGF mRNA by sequencing compared with basal level, going as high as 29.2% and 27.9% for patients 016 (metastatic adenocarcinoma of the tongue) and 017 (metastatic ovarian cancer), respectively (Fig. 2A; \( P < 0.000001 \)). The average of 3 separate measurements conducted on the same samples for these 2 patients was 19.9%.

### Table 3. Drug levels and changes in target mRNA in tumor biopsies after first dose

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Dose, mg/kg</th>
<th>Tumor type (biopsy site, day of postdose biopsy)</th>
<th>Viable tumor</th>
<th>Liver</th>
<th>Fibrosis/necrosis</th>
<th>Drug levels* (ng/g tissue)</th>
<th>Change in target mRNA (qRT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VEGF siRNA</td>
<td>KSP siRNA</td>
</tr>
<tr>
<td>007</td>
<td>0.40</td>
<td>Colorectal (liver, d7)</td>
<td>17</td>
<td>80</td>
<td>3</td>
<td>25.7</td>
<td>12.5</td>
</tr>
<tr>
<td>017</td>
<td>0.40</td>
<td>Ovarian (liver, d2)</td>
<td>0</td>
<td>95</td>
<td>5</td>
<td>28.9</td>
<td>17.2</td>
</tr>
<tr>
<td>019</td>
<td>0.70</td>
<td>Colorectal (liver, d2)</td>
<td>20</td>
<td>5</td>
<td>75</td>
<td>142</td>
<td>73.3</td>
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<tr>
<td>022</td>
<td>1.00</td>
<td>Colorectal (adrenal, d2)</td>
<td>10</td>
<td>0</td>
<td>78e</td>
<td>9.8</td>
<td>3.8</td>
</tr>
<tr>
<td>025</td>
<td>1.00</td>
<td>Sarcoma (muscle, d2)</td>
<td>96</td>
<td>0</td>
<td>4</td>
<td>0.45</td>
<td>0.40</td>
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<td>033</td>
<td>1.00</td>
<td>Colorectal (liver, d3)</td>
<td>56</td>
<td>0</td>
<td>35e</td>
<td>5.4</td>
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<td>041</td>
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<td>Renal cell (liver, d2)</td>
<td>70</td>
<td>15</td>
<td>15</td>
<td>6.0</td>
<td>6.8</td>
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<tr>
<td>042</td>
<td>1.00</td>
<td>Uveal melanoma (liver, d2)</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>7.0</td>
<td>5.8</td>
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<tr>
<td>026</td>
<td>1.25</td>
<td>Colorectal (liver, d6)</td>
<td>14</td>
<td>0</td>
<td>71e</td>
<td>0.32</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td>031</td>
<td>1.25</td>
<td>Ovarian (abdomen, d4)</td>
<td>30</td>
<td>0</td>
<td>70</td>
<td>4.9</td>
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<tr>
<td>035</td>
<td>1.25</td>
<td>Ovarian (lymph node, d5)</td>
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<td>0</td>
<td>60</td>
<td>20.4</td>
<td>20.3</td>
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<tr>
<td>032</td>
<td>1.50</td>
<td>Small bowel (liver, d6)</td>
<td>42</td>
<td>0</td>
<td>58</td>
<td>4.9</td>
<td>3.6</td>
</tr>
</tbody>
</table>

NOTE: N/A indicates no sample for analysis.

*Measured by qRT-PCR; LLOQ (lower limit of quantitation) = 0.14 ng/g tissue. All pretreatment biopsy samples were <LLOQ.

*Remaining 12% was normal adrenal (6%) and fat (6%).

*Remaining 9% was skeletal muscle.

*Remaining 15% was skeletal muscle.

\( P \) values by Relative Expression Analysis Tool (REST) analysis: Pt 007 VEGF: \( P < 0.001 \); Pt 031 VEGF: \( P = 0.001 \); Pt 035 VEGF: \( P < 0.001 \); KSP: \( P = 0.015 \).
Clinical Activity of an RNA Interference Therapeutic in Cancer Patients

Figure 2. Demonstration of RNAi in liver biopsies. A, sequencing from VEGF 5′ RACE analysis. The predicted specific VEGF mRNA cleavage product is indicated by the cyan bar; other VEGF fragments are shown in orange. *, P values compared with basal or predose level derived from t test. Liver, normal liver; CRC, colorectal cancer metastasis; patient 016, adenocarcinoma of the tongue (had pre- and posttreatment biopsies); patient 017, ovarian cancer (had posttreatment biopsy only); pre, pretreatment with ALN-VSP; post, 2 days after treatment with dose #1 ALN-VSP. B, Agilent microfluidic bioanalyzer DNA 1000 results from VEGF 5′ RACE second-round PCR conducted on untreated normal liver and on liver tumor biopsies from patients 016 and 017. Arrow points to predicted specific VEGF (230 bp) mRNA cleavage product.

and 23.9%, respectively (P = 0.019 and 0.007 by ANOVA). This increase in specific cleavage product was also shown by microfluidic chip analysis of the second-round PCR products (Fig. 2B). Neither of these patients had material available for mRNA quantitation by qRT-PCR. A third patient with an ovarian cancer peritoneal metastasis that contained 30% viable tumor, who was treated at 1.25 mg/kg and biopsied 96 hours after dosing, had a more modest average increase in the specific cleavage product for VEGF mRNA (4.3%, third highest among all patients analyzed) that did not reach statistical significance relative to basal level and was not seen on microfluidic chip analysis (data not shown). Measurement of VEGF mRNA by qRT-PCR in this same patient (031) showed a 74% reduction relative to pretreatment biopsy (P = 0.001; Table 3), and a liver metastasis showed a 64% reduction in K\textsubscript{\text{tumor}} after treatment as measured by DCE-MRI. Samples from 2 additional patients (035 and 007, with ovarian cancer lymph node metastasis and colorectal cancer liver metastases, respectively) biopsied 5 and 7 days after treatment showed 37% and 20% reductions in VEGF mRNA (P < 0.001; Table 3), respectively, but were negative for specific cleavage product by the 5′ RACE assay. Although these results provide evidence for VEGF mRNA downregulation by ALN-VSP in biopsies of both hepatic and extrahepatic tumors, the presence of normal liver and/or necrosis/fibrosis in these samples does not permit a determination of whether target downregulation was occurring in viable tumor tissue.

None of the 15 posttreatment biopsies were positive by KSP 5′ RACE; this result was not unexpected, as the levels of KSP mRNA in banked tumor and normal liver were substantially lower than VEGF mRNA (Supplementary Fig. S5), making detection of the specific cleavage product in biopsies with small amounts of viable tumor more difficult. However, the biopsy of the ovarian cancer lymph node metastasis that showed the 37% reduction in VEGF mRNA also showed a 25% reduction in KSP mRNA (P = 0.015; Table 3). Unipolar mitotic spindles were not seen in tumor biopsies. In contrast with the rapidly growing Hep3B tumors with synchronously dividing cells in the mouse orthotopic liver tumor model, the more slowly growing tumors in patients treated with ALN-VSP rarely had any identifiable mitoses in the small amounts of viable tumor present in most biopsies (data not shown). This finding was consistent with the 2-log lower KSP mRNA expression in banked tumors relative to Hep3B (Supplementary Fig. S5) and may have limited the ability to detect tumor cell monoasters indicative of an anti-KSP effect. Alternatively, the absence of monoasters may have been due to inadequate drug penetration into tumor and/or insufficient tumor cell uptake of the KSP siRNA.

Safety and Pharmacokinetic Profile

ALN-VSP was generally well tolerated, with predominantly low-grade fatigue/asthenia, nausea/vomiting, and fever
occurring in 15% to 24% of patients without any clear dose dependence (Supplementary Table S3). No clinically significant changes in liver function tests were observed in 40 of 41 patients, including 1 patient who received 50 doses at 0.7 mg/kg and 6 others dosed at 0.4 to 1.0 mg/kg who also went onto the extension study and received more than 8 doses. One patient with a PNET and more than 70% liver involvement with metastatic disease who had undergone prior splenectomy and partial hepatectomy tolerated the first dose well but developed hepatic failure several days after the second dose of ALN-VSP at 0.7 mg/kg and subsequently died. A CT scan conducted at the time of liver failure showed an increase in tumor necrosis relative to the pretreatment scan. As this patient’s DCE-MRI after the first dose showed a 50% to 60% decrease in $K_{trans}$ in the 3 liver tumors evaluated (Fig. 1C), overwhelming anti-VEGF-mediated tumor necrosis was one possible explanation for the liver failure, in addition to disease progression and/or drug-induced injury to the small amount of remaining normal liver. The prior splenectomy and partial hepatectomy were unique to this patient among all those enrolled onto the study. Given the biodistribution of LNPs to liver and spleen, this could have contributed to the liver toxicity that was not seen in any other subjects treated at doses as high as 1.5 mg/kg. In addition, as an increase in hepatic VEGF production has been observed following liver resection and is thought to be involved in liver regeneration (36, 37), it is possible that this patient’s liver may have been less able to tolerate local VEGF inhibition by ALN-VSP. In response to this serious adverse event, the protocol was subsequently amended to exclude patients with more than 50% liver involvement with tumor or prior splenectomy. Other dose-limiting toxicities (DLT) occurring during the study included transient grade 3 thrombocytopenia in 2 of 11 patients treated at 1.25 mg/kg (first episode during dose escalation and a second on the expansion phase, neither of which required platelet transfusion) and transient grade 3 hypokalemia in 1 patient treated at 1.5 mg/kg (Supplementary Table S4).

IRRs occurred after the first dose in 15% of patients dosed at $\geq$0.4 mg/kg and were readily managed by temporarily interrupting the infusion and giving the remainder of the dose over 30 to 60 minutes. These IRRs typically occurred within the first minutes of the infusion and usually included some combination of facial flushing, chest tightness, back/abdominal pain, elevated heart rate, or sweating. While increases in Bb complement were seen in most patients treated at $\geq$0.2 mg/kg, those patients with IRRs at 0.4 to 1.25 mg/kg tended to have more Bb induction (Fig. 3A). Total C3, C4, and CH50 did not change in patients with IRRs. Dose-dependent proinflammatory cytokine induction peaking after either the first or third dose was first observed at 0.4 mg/kg, where several patients were noted to have a modest, transient increase in serum IP-10 and interleukin (IL)-IRA peaking at 6 hours postdose and normalizing by 24 hours. Additional cytokines seen at $\geq$0.7 mg/kg that also peaked at 6 hours and normalized by 24 hours postdose included IL-6 (Fig. 3B), granulocyte colony-stimulating factor (G-CSF) and, less consistently, TNF-α. Cytokines not induced included IL-1β, IFN-α, and IFN-γ, with IFN-α being most commonly associated with Toll-like receptor (TLR) activation by single- or double-stranded RNA (18–20). Symptoms related to cytokine induction (grade 1–2 chills/rigors) were seen predominantly at 1.25 mg/kg in patients with peak IL-6 elevations of $\geq$1,000 pg/mL (Fig. 3B). These symptoms typically occurred 4 to 8 hours after the infusion, around the time of peak cytokine induction. There was no cytokine induction in the patient with endometrial cancer with the complete response treated at 0.7 mg/kg. In light of the DLTs seen at 1.25 and 1.5 mg/kg and the magnitude of cytokine induction with associated symptoms occurring at 1.25 mg/kg, the recommended phase II dose of ALN-VSP was determined to be 1.0 mg/kg.

Analysis of plasma pharmacokinetics for the VEGF and KSP siRNAs showed that maximum concentration ($C_{max}$) and area under the curve (AUC) were similar for both and dose-proportional (Fig. 4), with similar profiles after the first and third doses and no evidence of accumulation (data not shown). Approximately 97% of the siRNA detected in plasma was encapsulated. The pharmacokinetic profile in man was similar to what was observed in non-human primates (Supplementary Fig. S6).
Clinical Activity of an RNA Interference Therapeutic in Cancer Patients

DISCUSSION

The results of this first-in-humans study using LNP-formulated siRNAs to treat human disease have established the safety of chronic i.v. dosing in patients with advanced cancer and liver involvement. The study has also demonstrated the ability to measure drug in hepatic and extrahepatic tumor biopsies and provided evidence for RNAi-mediated target mRNA cleavage in liver, on-target pharmacodynamic effects in liver metastases, and antitumor activity at both hepatic and extrahepatic sites of disease, including a complete response.

From a safety standpoint, ALN-VSP was generally well tolerated, with an adverse event profile that compares favorably with chemotherapy and with other orally or intravenously administered targeted therapies in oncology. The IRRs seen in a minority of patients dosed at \( \geq 0.4 \) mg/kg were readily managed by slowing the infusion rate. Although C3a and C5a were not measured, the IRRs occurring with ALN-VSP seemed to be complement mediated, as they were not temporally related to cytokine induction and were associated with higher induction of Bb complement. Transient cytokine induction and mild to moderate chills and rigors occurring in a delayed manner after completion of dosing, while not DLTs, were seen in some patients predominantly at the higher doses of 1.0 and 1.25 mg/kg. Although the 2 siRNAs were chemically modified with 2'-O-methyl groups to minimize immunostimulation through RNA sensor pathways including TLRs (18–20), it is possible that either the siRNAs or one or more of the lipid components were involved in proinflammatory cytokine induction. Although the cause of the transient grade 3 thrombocytopenia occurring in 2 patients at 1.25 mg/kg is not known, the rapid onset within days of dosing and the swift recovery, as well as the absence of other cytopenias, makes temporary platelet activation and/or sequestration far more likely than marrow suppression. Notably, toxicities seen with systemic VEGF and KSP inhibition, such as hypertension/proteinuria (38–40) and myelosuppression/diarrhea (25, 26), respectively, were not observed with ALN-VSP. Although this could reflect a lower level of activity compared with antibodies and/or small molecules targeting these proteins, it could also be due to the more limited biodistribution of the LNP formulation to liver, spleen, and tumors, which is consistent with the absence of such toxicities in preclinical monkey toxicology studies using high doses of ALN-VSP. The one instance of hepatotoxicity seen in the patient dosed at 0.7 mg/kg with a particularly heavy tumor burden in the liver
who had undergone prior partial hepatectomy and splenectomy was of unclear etiology and underscores the importance of further evaluating safety across a range of disease presentations in future studies of patients with cancer involving the liver. However, it is notable that the liver safety profile was benign in the other 40 patients treated during the study at doses as high as 1.5 mg/kg, including those on the extension study who received biweekly treatments for an average of 11.3 months (with 2 patients at 0.7 and 1.0 mg/kg treated for 26 and 18 months, respectively).

Although the ALN-VSP phase I trial did require that patients have at least one measurable tumor in the liver, a notable finding of the study was the detection of drug in both hepatic and extrahepatic tumor biopsies. Both siRNAs in ALN-VSP were detected in liver tumor biopsies and in metastases involving the adrenal gland, lymph nodes, and abdominal cavity, across multiple different tumor types. In several instances, these biopsies were composed entirely of viable tumor, thereby showing that the drug was delivered to the tumor itself. However, other biopsies contained varying amounts of necrosis and fibrosis or normal tissue in addition to tumor, and therefore localization to the tumor could not be determined in those samples. The demonstration of RNAi-mediated VEGF mRNA cleavage in the liver of 2 patients, albeit in biopsy samples that predominantly showed normal liver with little or no tumor, establishes proof of mechanism for RNAi in humans using this novel siRNA-LNP formulation. The demonstration of VEGF mRNA downregulation in a liver metastasis from 1 patient and in extrahepatic metastases from 2 patients, though not conclusive for target downregulation in tumor due to the presence of other tissue types in the biopsy samples, nonetheless provides additional examples of VEGF mRNA modulation in vivo by ALN-VSP supportive of proof of mechanism for RNAi.

A possible clinical correlate of the observed changes in VEGF mRNA was the substantial decrease in tumor blood flow seen on DCE-MRI in nearly half of the patients with evaluable liver tumors. Although qualitatively these changes in blood flow resembled the reported changes observed with anti-VEGF antibodies and small-molecule inhibitors of the VEGFR (41, 42), no dose response was observed. One therefore cannot exclude the possibility that these findings were the result of a nonspecific effect of the LNP formulation or premedication regimen rather than a specific effect of the VEGF siRNA in ALN-VSP. Although we were not able to detect tumor cell monoasters indicative of an anti-KSP effect in tumor biopsies, changes in spleen volume were observed that were indicative of an anti-KSP effect based on preclinical data in monkeys. This finding, combined with the demonstration of KSP mRNA reduction in an extrahepatic tumor biopsy, suggests that pharmacodynamic changes consistent with both VEGF and KSP inhibition were observed with ALN-VSP.

Major tumor responses, especially complete responses, are infrequently seen in phase I studies involving heavily pretreated patients with progressive disease. Therefore, it is notable that the ALN-VSP phase I trial included a complete response in a patient with nodal and extensive liver metastases, as well as prolonged disease stabilization for as long as 1 to 1.5 years in patients with hepatic and extrahepatic metastases. With respect to the mechanism underlying the observed antitumor activity, there is no evidence that this was an off-target effect of the drug. While transient cytokine induction was observed after the first and third doses of drug in some patients treated at the higher dose levels, this change was not observed in the patient who achieved the complete response. Therefore, it is unlikely that the tumor regression resulted from an antitumor immune response. In the mouse Hep3B tumor model, a control siRNA formulated in the same LNP as ALN-VSP had no antitumor activity, further showing that the activity of ALN-VSP was not due to an off-target effect of the LNP. Given the heterogeneity of tumor types and prior therapies (including prior anti-VEGF therapies) and the relatively small number of patients treated across various dose levels, it is unlikely that this phase I study would have been able to establish correlations between on-target pharmacodynamic changes and clinical outcome. Subsequent phase II trials with ALN-VSP focusing on a single tumor type, enrolling patients with fewer prior therapies, and treating all patients at the same active dose level will be better suited for examining the relationship between target modulation and clinical response to therapy.

Overall, these findings show the safety, pharmacokinetics, RNAi mechanism of action, and clinical activity with a novel first-in-class RNAi therapeutic in humans. These findings build on the results previously reported with CALAA-01 (9), a targeted cyclodextrin-based nanoparticle similar in size to ALN-VSP that contains an siRNA targeting ribonucleoside-diphosphate reductase subunit M2 (RRM2). In that report, CALAA-01 was shown to localize in cutaneous melanoma metastases following i.v. administration and to mediate target mRNA cleavage and downregulation. Here, the data with ALN-VSP suggest that dual targeting of VEGF and KSP was attained at both the molecular and clinical level. This study also provides the basis for further development of ALN-VSP in malignancies responsive to anti-VEGF drugs, such as endometrial cancer (43), PNET (32, 39), RCC (30, 38) and hepatocellular carcinoma (44), in which more effective and better-tolerated therapies are needed.

METHODS

Animal Experiments

Intrahepatic tumors were generated by injection of 1 × 10⁶ human Hep3B hepatocellular carcinoma cells (American Type Culture Collection; catalog no. HB-8064; cells were authenticated through analysis of protein expression, presence of HepB viral DNA by PCR, and demonstration of tumorigenicity in nude mice), suspended in 0.025 mL PBS, directly into the livers of SCID/beige mice. Surgery was carried out under isoflurane anesthesia and according to Institutional Animal Care and Use guidelines. Tumor growth was monitored using AFP ELISA (Sierra Resources International). Animals were randomly selected into groups with similar mean tumor size before treatment, and only tumor-bearing animals were used in experiments. To determine the antitumor effect of ALN-VSP, tumor-bearing animals began treatment 4 weeks after implantation. Animals were euthanized on the basis of humane endpoints. At the end of the study all remaining animals were euthanized. For histology and imaging, tumors were fixed in buffered formalin and paraffin embedded. Whole tumor sections were stained with hematoxylin

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Clinical Activity of an RNA Interference Therapeutic in Cancer Patients

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**Bioanalytical Methods**

RNA was purified from blinded biopsy samples following the standard RNAeasy Micro Kit (Qiagen; catalog no. 64004) protocol with minor modifications. Quality and quantity of RNA was assessed with a Bioanalyzer2100 (Agilent; catalog no. G2940CA).

qRT-PCR was conducted using LightCycler 480 Probe Master Mix (Roche Applied Science; catalog no. 04887301001) and VEGF-A (Life Technologies; catalog no. Hs00173462_m1), KIF11 (Life Technologies; catalog no. Hs00189698_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Life Technologies; catalog no. 4326317E) or CEAAMS (Life Technologies; catalog no. Is02377075_m1) gene-specific TaqMan probes on a LightCycler 480 system (Roche). qRT-PCR data analysis was conducted using the Relative Expression Analysis Tool (REST). 5′ RACE was conducted for either KIF11 or VEGFA as per the GeneRacer Kit protocol (Life Technologies; catalog no. L1500-01) with minor modifications. Standard Illumina genomic sequencing adaptors were added to the second round 5′ RACE PCR primers and sequenced using a custom Illumina sequencing primer. Data were processed using custom Perl programs. Sequences were aligned to the appropriate transcript (VEGF-A = NM_001025368.3 and KIF11 = NM_004523.3). The cleavage position in KIF11 is 1249 and for VEGF-A is 1393. See Supplementary Methods for more details on 5′ RACE assay.

**Phase I Clinical Trial**

Patients ages 18 years or older with advanced solid tumors confirmed by histologic or cytologic examination that had recurrent or progressed following standard therapy, and have stable disease or better by RECIST, in addition to American Cancer Society (ACS) approved and registered on Clinical Trials.gov (NCT01158079). Written informed consent was obtained from patients. To be eligible for the extension study (NCT01158079), patients had to have completed 4 cycles of treatment and have stable disease or better by RECIST, in addition to meeting the same laboratory and performance status eligibility criteria. Patients were monitored for adverse events using version 3.0 of the National Cancer Institute Common Toxicity Criteria.

**Cytokines and Complement**

Cytokines were measured in serum samples obtained before dosing and 2, 6, and 24 hours after infusion for the first and third doses. Measurements of IFN-α, IFN-γ, IL-6, IL-12, TNF-α, IL-18, IL-1RA, G-CSF, and IP-10 were conducted at Charles River Laboratories. Complement factors C3, C4, CH50, and Bb were measured before dosing and 30 minutes, 2 hours, and 24 hours after infusion for the first and third doses. C3, C4, and CH50 were measured locally; Bb measurement was conducted at Charles River Laboratories.

**Pharmacokinetics**

For pharmacokinetic analyses, plasma and plasma filtrate from specified time points were analyzed for total and unencapsulated siRNA, respectively, using a validated hybridization ELISA for each of the 2 siRNAs (Charles River Laboratories). Analysis of the concentration–time data was conducted by Charles River Laboratories, and the pharmacokinetic profile of each patient was characterized by noncompartamental analysis of each siRNA plasma concentration using validated computer software (WinNonlin, version 3.2, Pharsight Corp.).

**Tumor Biopsies**

Core biopsies of tumors were carried out under CT guidance. Where possible, 3 separate cores were obtained from the same tumor at each time point. Two cores obtained for siRNA quantitation and for VEGF/KSP qPCR and 5′ RACE were snap frozen in liquid nitrogen and sent to Alnylam Pharmaceuticals for processing; the third core was fixed in formalin and sent to DCL Medical Laboratories, where it was imbedded in paraffin before sectioning and staining with H&E for histopathologic evaluation.

**Disclosure of Potential Conflicts of Interest**

R.M. Hutabarat and J. Celesky are employed as Senior Directors for Alnylam Pharmaceutical, Inc. S.V. Nochur is employed as Vice President for Regulatory Affairs, and J.A. Gollob is employed as Vice President for Clinical Research, both for Alnylam Pharmaceuticals, Inc. C. Gamba-Vitalo has ownership interest (including patents) in Alnylam 401K. A.K. Vaishnaw is employed as Chief Medical Officer for Alnylam Pharmaceuticals, Inc. G. Weiss has received honoraria and served on the speakers’ bureau for Genentech and Pfizer. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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