First-in-Man Trial of an RNA Interference Therapeutic Targeting VEGF and KSP in Cancer Patients with Liver Involvement


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ABSTRACT
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 (LNPs) have proven effective in delivery of siRNAs to liver and tumors in animals. To examine the activity and safety of LNP-formulated siRNAs in man, we initiated a trial of ALN-VSP, an LNP formulation of siRNAs targeting VEGF and KSP, in cancer patients. Here we demonstrate detection of drug in tumor biopsies, siRNA-mediated mRNA cleavage in liver, pharmacodynamics suggestive of target downregulation, and antitumor activity including complete regression of liver metastases in endometrial cancer. In addition, we show that bi-weekly intravenous administration of ALN-VSP was safe and well-tolerated. These data provide proof of concept for RNAi therapeutics in man and form the basis for further development in cancer.

SIGNIFICANCE
The findings in this report demonstrate safety, pharmacokinetics, RNAi mechanism of action, and clinical activity with a novel first-in-class LNP-formulated RNAi therapeutic in cancer patients. The ability to harness RNAi to facilitate specific multi-targeting, as well as increase the number of druggable targets, has important implications for future drug development in oncology.
INTRODUCTION

RNA interference (RNAi) is an endogenous cellular mechanism for controlling gene expression in which small interfering RNAs (siRNAs) bound to RISC (RNA-Induced Silencing Complex) 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). Since 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 cytosol. 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 (LNPs) 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 non-human primates (11-14). Delivery to tumors has also been demonstrated in murine orthotopic liver tumor and subcutaneous tumor models, where both mRNA silencing and on-target pharmacology were observed following intravenous dosing (17).

While results in animal models with LNPs are promising, it remains to be shown that this will translate in man into an effective way to deliver siRNAs for the treatment of human disease. In order to examine the activity and safety of LNP-formulated siRNAs in man, we initiated the first Phase I trial utilizing this approach to treat patients with advanced cancer and liver metastases.
RESULTS

ALN-VSP Composition and Preclinical Safety and Activity

ALN-VSP is comprised of an LNP containing two different siRNAs targeting vascular endothelial growth factor-A (VEGF-A) (18) and kinesin spindle protein (KSP, encoded by the KIF11 gene) (19) in a 1:1 molar ratio. Both siRNAs were chemically modified to reduce their immunostimulatory potential (20-22) (Suppl Figure 1). ALN-VSP has a particle diameter of 80-100 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 (23, 24), 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 (25, 26).

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 ALN-VSP every two weeks but not in control animals treated with the same dose of LNP containing an siRNA targeting luciferase (Suppl Table 1). These findings were indicative of an on-target siRNA effect rather than a non-specific effect of the LNP itself, and the lymphopenia reported in patients treated with a small molecule KSP inhibitor (27, 28) 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 SCID/beige mice implanted with the Hep3B human hepatocellular carcinoma cells, the intravenous 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 dosing with 4 mg/kg. This 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 (13-16) (Suppl Figure 2A). 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 (Suppl Figure 2B), consistent with KSP inhibition. In mice given repetitive doses of the LNP-formulated VEGF siRNA (LNP-VEGF), both a reduction in tumor hemorrhage and microvascular density were seen to a degree comparable to animals treated with the anti-VEGF antibody bevacizumab (Suppl Figure 2C). Following these demonstrations of anti-KSP and anti-VEGF pharmacodynamics, the antitumor activity of ALN-VSP administered as repeat 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 (Suppl Figure 2D). In addition to having an impact on hepatic tumors, parenterally administered ALN-VSP also has activity against extrahepatic tumors, as demonstrated by the detection of tumor cell monoasters following ALN-VSP treatment in mice with abdominal peritoneal Hep3B tumor implants (Suppl Figures 3A and 3B).

Phase I Study Design and Patient Characteristics

The dose escalation phase of the study utilized 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 intravenous (IV) infusion via a controlled infusion.
device using an extension set with a 1.2 micron filter every two weeks, with a cycle of therapy defined as two doses given over one month (Suppl Figure 4). All patients were premedicated prior to each dose with dexamethasone 8 mg orally (PO) the night before and dexamethasone 20 mg IV, acetaminophen 650 mg PO, diphenhydramine 50 mg IV (or hydroxyzine 25 mg PO) and either ranitidine 50 mg or famotidine 20 mg IV 30 minutes prior to infusion to reduce the risk of infusion-related reactions (IRRs) observed with other liposomal products (29). Tumor measurements by computed tomography (CT) scan were performed after every 2 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 30 on the dose escalation phase treated at 0.1-1.5 mg/kg and 11 on the expansion phase treated at 1.0 (N=6) or 1.25 mg/kg (N=5, Table 1). Almost all patients were heavily pretreated with either chemotherapy and/or anti-VEGF/VEGFR receptor (VEGFR) agents. The majority had both hepatic and extrahepatic tumors. 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 0.4-1.0 mg/kg whose disease did not progress after the first 4 months went onto the extension study (Suppl Table 2) and were treated for an average of 11.3 months (range 5-26); 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 RECIST [Response Evaluation Criteria in Solid Tumors] as disappearance of all target and non-target
lesions) occurred in a patient with endometrial cancer and multiple hepatic metastases (Figure 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 dosed 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 8-12 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 magnetic resonance imaging (DCE-MRI) was performed at baseline and twice during the week following 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 were required to have at least one liver tumor measuring ≥2 cm in diameter. Both transfer constant (Ktrans) 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% peak average reduction in Ktrans (Figure 1B), although there was no evidence of dose-dependence. Changes in Ktrans and IAUGC tended to occur in parallel, and were often seen within days of the first dose in both large and small tumors (Figure 1C).
To determine whether ALN-VSP was capable of causing changes in spleen size observed in monkeys, serial measurements of spleen volume were undertaken retrospectively in 25 evaluable patients who had abdominal CT scans performed 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), there was an average reduction of \(-37\pm25\%\) (range +8 to -92\%) in patients dosed at 0.4-1.5 mg/kg (Figure 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.

While there was no overall correlation between change in Ktrans or spleen volume and tumor response, the endometrial cancer patient with the complete response had a >50\% decrease in Ktrans and 90\% decrease in spleen size.

**RNAi Proof of Mechanism**

Voluntary CT-guided core needle tumor biopsies were performed in 15 patients dosed at 0.4-1.5 mg/kg to measure drug levels and look for evidence of RNAi. Biopsies were taken prior to the first dose of ALN-VSP and then again 2-7 days post-dose. Biopsied metastatic tumors were hepatic in 11 patients and extrahepatic in 4 patients. The biopsies were notable for the considerable degree of interpatient 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 twelve patients who had biopsies evaluable for both VEGF and KSP siRNAs using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (Table 3) (35), all had detectable VEGF siRNA post-dose (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 96-100% viable tumor (#025 with sarcoma muscle metastasis and #042 with melanoma liver metastasis), thereby suggesting drug delivery to tumor itself. As 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 performed 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 pre-treatment 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 post-treatment biopsies, two patients biopsied 48 hours after dosing whose liver tumor biopsies were comprised 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 to 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 (Figure 2A, p<0.000001). The average of 3 separate measurements performed on the same samples for these two patients was 19.9% and 23.9%, respectively (p-values of 0.019 and 0.007 by ANOVA). This increase in specific cleavage product was also demonstrated by microfluidic chip analysis of the second-round PCR products (Figure 2B). Neither of these patients had material available for mRNA quantitation by qPCR. 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 qPCR 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 Ktrans post-dose by DCE-MRI. Samples from two additional patients (#035
and #007 with ovarian cancer lymph node metastasis and colorectal cancer liver metastases,
respectively) biopsied 5 and 7 days post-dose had 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. While 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 post-treatment biopsies were positive by KSP 5’ RACE; this was not unexpected,
as the levels of KSP mRNA in banked tumor and normal liver were substantially lower than
VEGF mRNA (Suppl Figure 5), 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 to 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 was consistent with the 2-log lower KSP mRNA expression
in banked tumors relative to Hep3B (Suppl Figure 5), 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 anti-KSP siRNA.

Safety and PK Profile

ALN-VSP was generally well-tolerated, with predominantly low-grade fatigue/asthenia,
nausea/vomiting, and fever occurring in 15-24% of patients without any clear dose-dependence
(Suppl Table 3). No clinically significant changes in liver function tests were observed in 40 of
41 patients, including one patient who received 50 doses at 0.7 mg/kg and six others dosed at
0.4-1.0 mg/kg who also went onto the extension study and received >8 doses. One patient with a
PNET and over 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
performed 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 50-60% decrease in
K\text{trans} in the three liver tumors evaluated (Figure 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 led to a greater
exposure of the remaining liver to drug and 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 >50% liver involvement with tumor
or prior splenectomy. Other dose-limiting toxicities (DLTs) occurring on the study included
transient grade 3 thrombocytopenia in 2 of 11 patients treated at 1.25 mg/kg (one episode during
dose escalation and a second on the expansion phase, neither of which required platelet
transfusion) and transient grade 3 hypokalemia in one patient treated at 1.5 mg/kg (Suppl Table
4).

Infusion-related reactions (IRRs) occurred after the first dose in 15% of patients dosed at ≥0.4
mg/kg and were readily managed by temporarily interrupting the infusion and giving the
remainder of the dose over 30-60 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 ≥0.2 mg/kg, those patients with IRRs at 0.4-1.25 mg/kg tended to
have more Bb induction (Figure 3A). Total C3, C4 and CH50 did not change in patients with
IRRs. Dose-dependent pro-inflammatory 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 rise in serum IP-10 and IL-1RA peaking at 6 hrs post-dose and normalizing by 24 hrs.
Additional cytokines seen at ≥0.7 mg/kg that also peaked at 6 hours and normalized by 24 hours
post-dose included IL-6 (Figure 3B), 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 (20-22). 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 ≥1000 pg/mL (Figure 3B). These symptoms typically occurred 4-8 hours after the infusion, around the time of peak cytokine induction. There was no cytokine induction in the endometrial cancer patient 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 PK for the VEGF and KSP siRNAs showed that maximum concentration (Cmax) and area under the curve (AUC) were similar for both and dose-proportional (Figure 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 PK profile in man was similar to what was observed in non-human primates (Suppl Figure 6).
DISCUSSION

The results of this first-in-man study using LNP-formulated siRNAs to treat human disease have established the safety of chronic intravenous dosing in patients with advanced cancer and liver involvement, 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 AE profile that compares favorably to chemotherapy and to other orally or intravenously administered targeted therapies in oncology. The IRRs seen in a minority of patients dosed at ≥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 appeared 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/rigors occurring in a delayed manner after completion of dosing, while not dose-limiting toxicities, were seen in some patients predominantly at the higher doses of 1.0 and 1.25 mg/kg. Although the two siRNAs were chemically modified with 2’-O-methyl groups to minimize immunostimulation through RNA sensor pathways including TLRs (20-22), it is possible that either the siRNAs and/or one or more of the lipid components were involved in proinflammatory cytokine induction. While the etiology 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 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 (27, 28), respectively, were not observed with ALN-VSP. While this could reflect a lower level of activity compared to 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 quite benign in the other 40 patients treated on 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 two patients at 0.7 and 1.0 mg/kg treated for 26 and 18 months, respectively).

While the ALN-VSP Phase 1 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 adrenal gland, lymph nodes, and the abdominal cavity, across multiple different tumor types. While in several instances these biopsies were comprised entirely of viable tumor, thereby showing that drug was delivered to tumor itself, all other biopsies contained varying amounts of necrosis/fibrosis and/or normal tissue in addition to tumor, and therefore localization to 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 were predominantly normal liver with little to no tumor, establishes proof of mechanism for RNAi in
man using this novel siRNA-LNP formulation. The demonstration of VEGF mRNA
downregulation in a liver metastasis from 1 patient and in extrahepatic metastases from two
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. While qualitatively these changes in blood flow resembled the reported changes
observed with anti-VEGF antibodies and small molecule inhibitors of the VEGF receptor (41, 42),
there was no dose response. One therefore cannot exclude the possibility that these findings
were the result of a non-specific effect of the LNP formulation or premedication regimen rather
than a specific effect of the anti-VEGF siRNA in ALN-VSP. While 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 1½ years in patients with
hepatic and extrahepatic metastases. Regarding 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 following the first and third doses of drug was observed in some patients treated at the higher dose levels, this was not seen in the patient who achieved the complete response. Therefore, it is unlikely that the tumor regression was the result of 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 demonstrating 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 study would have been able to establish correlations between on-target pharmacodynamic changes and clinical outcome on this sort of Phase I trial. 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 in patients demonstrate safety, pharmacokinetics, RNAi mechanism of action, and clinical activity with a novel first-in-class RNAi therapeutic in man. This builds 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 intravenous administration and 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), renal cell cancer (30, 38) and hepatocellular carcinoma (44), where there is continued high unmet need for more effective and better tolerated therapies.
METHODS

Animal Experiments

Intrahepatic tumors were generated by injection of $1 \times 10^6$ human Hep3B hepatocellular carcinoma cells (American Type Culture Collection, Manassas, Virginia, USA; catalog #HB-8064; cells authenticated through analysis of protein expression, presence of HepB viral DNA by PCR, and demonstration of tumorigenicity in nude mice), suspended in 0.025 cc PBS, directly into the livers of SCID/beige mice. Surgery was performed under isofluorane anesthesia and according to institutional animal care and use guidelines. Tumor growth was monitored using AFP ELISA (Sierra Resources International, Santa Fe, NM, USA). 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 anti-tumor effect of ALN-VSP, tumor bearing animals were treated starting at 4 weeks post-implantation. Animals were euthanized based on 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 H&E to quantify monoasters or hemorrhage or with anti-CD34 (Abcam, Cambridge, MA, USA) to quantify microvasculature. Regions of intratumoral hemorrhage were outlined in H&E stained sections and total areas of hemorrhage were quantified in each tumor. CD34 stained areas were quantified as a percentage of total tumor area.

Bioanalytical Methods

RNA was purified from blinded biopsy samples following the standard RNAeasy micro (Qiagen, Valencia, CA, USA, Cat.No.74004) protocol with the minor modifications. Quality and quantity of RNA was assessed with a Bioanalyzer2000 (Agilent, Santa Clara, CA, USA, Cat.No.G2940CA). qPCR was performed using LightCycler® 480 Probe Master Mix (Roche...
Applied Science, Indianapolis, IN, USA, Cat. No. 04887301001) and VEGF-A (Life Technologies, Cat. No.Hs00173626_m1), KIF-11 (Life Technologies, Grand Island, NY, USA, Cat. No. Hs00189698_m1), GAPDH (Life Technologies, Cat. No. 4326317E) or CEACAM5 (Life Technologies, Cat. No. hs00237075_m1) gene specific Taqman probes on a LightCycler® 480 system (Roche). qPCR data analysis was performed using the Relative Expression Analysis Tool (REST). 5’ RACE was performed for either KIF11 or VEGF-A as per the GeneRacer kit protocol (Life Technologies, Cat.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 ≥18 years with histologically or cytologically confirmed advanced solid tumors that had recurred or progressed following standard therapy, had not responded to standard therapy, or for which there was no standard therapy, were eligible to participate in this study if they had at least one measurable tumor (≥1 cm by spiral CT or ≥2 cm by standard CT) in the liver. Other eligibility criteria included Eastern Cooperative Oncology Group performance status of 0-1, at least 28 days out from prior systemic therapy, radiotherapy, or major surgery, aspartate and alanine aminotransferase ≤2.5 times upper limit of normal, total bilirubin within normal limits, albumin >3.0 g/dL, international normalized ratio ≤1.2, absolute neutrophil count ≥1500 cells/mm³, platelet count ≥100,000 cells/mm³, hemoglobin ≥9 g/dL, and serum creatinine ≤1.5 times upper limit of normal. Patients were excluded if they had brain or leptomeningeal
metastases, known infection with hepatitis B or C or human immunodeficiency virus, >50% involvement of the liver by tumor, or had previously undergone splenectomy. There was no limit placed on number of prior therapies. The study was approved by each site’s Institutional Review Board and registered on ClinicalTrials.gov (NCT00882180). 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 NCI Common Toxicity Criteria.

**Cytokines and Complement**

Cytokines were measured in serum samples obtained pre-dose and 2, 6 and 24 hours post-infusion for the first and third doses. Measurements of IFN-α, IFN-γ, IL-6, IL-12, TNF-α, IL-1β, IL-1RA, G-CSF and IP-10 were performed at Charles River Laboratories (Montreal, Canada). Complement factors C3, C4, CH50 and Bb were measured pre-dose and 30 minutes, 2 and 24 hours post-infusion for the first and third doses. C3, C4 and CH50 were measured locally; Bb measurement was performed 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 enzyme-linked immunosorbent assay for each of the two siRNAs (Charles River Laboratories). Analysis of the concentration-time data was performed by Charles River Laboratories, and the PK profile of each patient characterized by non-compartmental analysis of each siRNA plasma concentration using validated computer software (WinNonlin, version 3.2, Pharsight Corp., Mountain View, California, USA).
**Tumor Biopsies**

Core biopsies of tumors were performed 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 (Cambridge, MA, USA) for processing; the third core was fixed in formalin and sent to DCL Medical Laboratories (Indianapolis, IN, USA), where it was imbedded in paraffin prior to sectioning and staining with hematoxylin and eosin for histopathologic evaluation.
REFERENCES


8. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA


Table 1. ALN-VSP Phase 1 Trial: Demographics and Dosing

<table>
<thead>
<tr>
<th>Demographic</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 # of prior regimens for 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>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 # of doses/patient</td>
<td>6.8 (range 1-50)</td>
</tr>
</tbody>
</table>

ECOG: Eastern Cooperative Oncology Group
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># 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>

SD: stable disease
CR: complete response
Table 3. Drug Levels and Changes in Target mRNA in Tumor Biopsies Post-First Dose

<table>
<thead>
<tr>
<th>Pt #</th>
<th>Dose (mg/kg)</th>
<th>Tumor Type (Biopsy Site, Day of Post-Dose Biopsy)</th>
<th>Post-Dose Biopsy (%)</th>
<th>Drug Levels* (ng/g tissue)</th>
<th>Change in Target mRNA (qPCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Viable Tumor</td>
<td>Liver</td>
<td>Fibrosis/Necrosis</td>
</tr>
<tr>
<td>007</td>
<td>0.40</td>
<td>Colorectal (liver, d7)</td>
<td>17</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>017</td>
<td>0.40</td>
<td>Ovarian (liver, d2)</td>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>019</td>
<td>0.70</td>
<td>Colorectal (liver, d2)</td>
<td>20</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>022</td>
<td>1.00</td>
<td>Colorectal (adrenal, d2)</td>
<td>10</td>
<td>0</td>
<td>78**</td>
</tr>
<tr>
<td>025</td>
<td>1.00</td>
<td>Sarcoma (muscle, d2)</td>
<td>96</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>033</td>
<td>1.00</td>
<td>Colorectal (liver, d3)</td>
<td>56</td>
<td>0</td>
<td>35†</td>
</tr>
<tr>
<td>041</td>
<td>1.00</td>
<td>Renal cell (liver, d2)</td>
<td>70</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>042</td>
<td>1.00</td>
<td>Uveal melanoma (liver, d2)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>026</td>
<td>1.25</td>
<td>Colorectal (liver, d6)</td>
<td>14</td>
<td>0</td>
<td>71†</td>
</tr>
<tr>
<td>031</td>
<td>1.25</td>
<td>Ovarian (abdomen, d4)</td>
<td>30</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>035</td>
<td>1.25</td>
<td>Ovarian (lymph node, d5)</td>
<td>40</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>032</td>
<td>1.50</td>
<td>Small bowel (liver, d6)</td>
<td>42</td>
<td>0</td>
<td>58</td>
</tr>
</tbody>
</table>

*Measured by qPCR, LLOQ (lower limit of quantitation) = 0.14 ng/g tissue. All pre-treatment 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 REST analysis:
Pt 007 VEGF: p<0.001
Pt 031 VEGF: p=0.001
Pt 035 VEGF: p<0.001
KSP: p=0.015
N/A: No sample for analysis
FIGURE LEGENDS

Figure 1. Clinical activity of ALN-VSP in cancer patients. (A) Complete response in endometrial cancer patient #021 with multiple liver metastases. CT images of metastatic tumors (black arrows) obtained prior to start of ALN-VSP (Pre-Treatment) and following 12 and 40 doses of drug. (B) Decrease in blood flow (Ktrans) in liver tumors of patients treated with ALN-VSP. For 24 patients with evaluable DCE-MRI scans, average peak change in Ktrans for one or more liver tumors occurring during the first week after Dose #1 shown for each patient by dose level. (C) DCE-MRI images from patient #012 with metastatic PNET treated at 0.7 mg/kg. Three tumors in right lobe of liver seen on abdominal MRI (top left panel) indicated by white arrows. Top right panel shows peak changes in Ktrans and IAUGC occurring during first week after Dose #1 in each tumor. Bottom panels show corresponding DCE-MRI images of tumor blood flow (indicated in red). (D) Decrease in spleen volume in patients treated with ALN-VSP. Spleen volume was measured on abdominal CT performed for tumor measurements in 25 evaluable patients with both pre- and post-treatment scans.

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 to basal or pre-dose level derived from T-test. Liver: normal liver; CRC: colorectal cancer metastasis; Patient 016: adenocarcinoma of the tongue (had pre- and post-treatment biopsies); Patient 017: ovarian cancer (had post-treatment biopsy only); Pre: pre-treatment with ALN-VSP; Post: 2-days post-treatment with Dose #1 ALN-VSP. (B) Agilent microfluidic bioanalyzer DNA 1000 results from VEGF 5’ RACE second round PCR performed 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.

**Figure 3. Complement and cytokines in patients treated with ALN-VSP.** Bb complement (A) and IL-6 (B) induction by dose level. Peak fold-increase following the first and third doses of ALN-VSP is shown for individual patients. P-values derived from linear regression used to model the parameter response as a function of dose level.

**Figure 4. ALN-VSP Phase I PK data.** Mean plasma PK results for KSP (left panel) and VEGF (right panel) siRNAs shown for each dose level following the first dose of ALN-VSP.
Tabernero Figure 1

a) Pre-Treatment, After 12 Doses ALN-VSP, After 40 Doses ALN-VSP

b) 

| # of Tumors | 1 | 3 | 1 | 1 | 3 | 1 | 1 | 2 | 1 | 1 | 3 | 3 | 3 | 3 | 3 | 1 | 3 | 1 | 1 | 1 |
| 0.1 n=2    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 0.2 n=1    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 0.4 n=4    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 0.7 n=6    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1.0 n=8    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1.25 n=3   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1.5 n=1    |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

Average Ktrans Change from Baseline (%)

Dose Level (mg/kg): 0.1 n=1, 0.2 n=2, 0.4 n=4, 0.7 n=6, 1.0 n=8, 1.25 n=3, 1.5 n=1

0.1 0.2 0.4 0.7 1.0 1.25 1.5

Pre-Treatment

Ktrans and IAUGC Change from Baseline (%)

T1 T2 T3

Baseline MRI, Coronal View

Patient 012

Ktrans

Baseline (BL) Pre-Dose

Day 4 Post-Dose 1

Day 7 Post-Dose 1

Ktrans2

Ktrans3

IAUGC2

IAUGC3

T: liver tumor number

Ktrans2/IAUGC2: Δ DCE-MRI #1 (BL) to DCE-MRI #2 (Day 4)

Ktrans3/IAUGC3: Δ DCE-MRI #1 (BL) to DCE-MRI #3 (Day 7)

c) 

d) 

# doses: 4 4 4 4 2 4 4 4 3 8 8 8 8 8 8 8 8 3 12 8 8 8 2 4 4

% Change in Spleen Volume

Dose Level (mg/kg): 0.1 n=1, 0.2 n=2, 0.4 n=4, 0.7 n=6, 1.0 n=8, 1.25 n=3, 1.5 n=1

Avg. ↓: 37%
Tabernero Figure 2

a

<table>
<thead>
<tr>
<th></th>
<th>% Total Reads</th>
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</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.102%</td>
</tr>
<tr>
<td>CRC</td>
<td>0.64%</td>
</tr>
<tr>
<td>Patient 016 Pre</td>
<td>1.41%</td>
</tr>
<tr>
<td>Patient 016 Post</td>
<td>29.2%</td>
</tr>
<tr>
<td>Patient 017 Post</td>
<td>27.9%</td>
</tr>
</tbody>
</table>

p<0.000001*

5' Location Along VEGF Transcript 3'

b

Ladder

Normal Liver

Pre 016

Post 017

400 300 200 150 100
peak Fold-Change Bb
Over Baseline

p=0.417 for dose response

Dose (mg/kg)

p<0.0001 for dose response

Dose (mg/kg)
Tabernero Figure 4

KSP siRNA

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>0.10 (n=3)</th>
<th>0.20 (n=3)</th>
<th>0.40 (n=5)</th>
<th>0.70 (n=7)</th>
<th>1.00 (n=4)</th>
<th>1.25 (n=5)</th>
<th>1.50 (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max} (μg/mL)</td>
<td>0.76 ± 0.36</td>
<td>2.26 ± 0.54</td>
<td>3.92 ± 1.12</td>
<td>7.99 ± 3.39</td>
<td>15.31 ± 5.35</td>
<td>21.94 ± 7.64</td>
<td>18.04</td>
</tr>
<tr>
<td>AUC_{0-last} (min*μg/mL)</td>
<td>30.83 ± 20.86</td>
<td>133.02 ± 46.66</td>
<td>252.38 ± 97.9</td>
<td>523.18 ± 306.18</td>
<td>923.59 ± 360.13</td>
<td>2035.74 ± 1100.08</td>
<td>1491.03</td>
</tr>
</tbody>
</table>

VEGF siRNA

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>0.10 (n=3)</th>
<th>0.20 (n=3)</th>
<th>0.40 (n=5)</th>
<th>0.70 (n=7)</th>
<th>1.00 (n=4)</th>
<th>1.25 (n=5)</th>
<th>1.50 (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max} (μg/mL)</td>
<td>0.86 ± 0.42</td>
<td>2.51 ± 0.56</td>
<td>4.31 ± 1.10</td>
<td>8.81 ± 2.75</td>
<td>15.84 ± 7.01</td>
<td>21.58 ± 6.80</td>
<td>18.19 ± 1.09</td>
</tr>
<tr>
<td>AUC_{0-last} (min*μg/mL)</td>
<td>69.95 ± 66.12</td>
<td>143.11 ± 58.58</td>
<td>272.21 ± 98.16</td>
<td>626.37 ± 272.66</td>
<td>984.26 ± 436.81</td>
<td>2006.58 ± 1113.54</td>
<td>1514.63 ± 150.24</td>
</tr>
</tbody>
</table>
First-in-Man Trial of an RNA Interference Therapeutic Targeting VEGF and KSP in Cancer Patients with Liver Involvement

Josep Tabernero, Geoffrey I. Shapiro, Patricia M. LoRusso, et al.

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