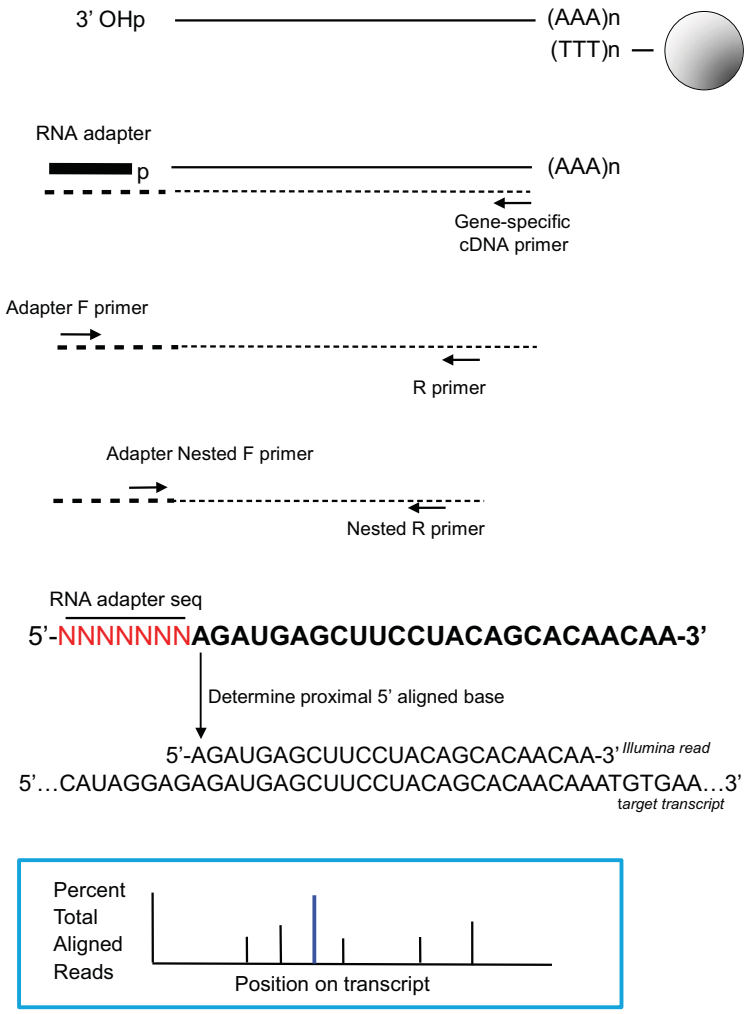
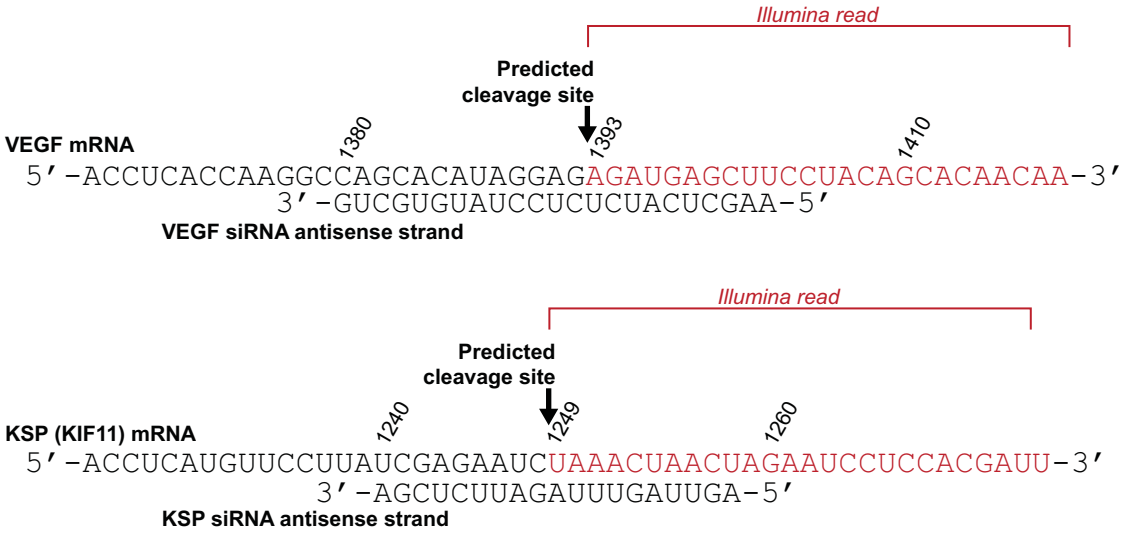
Item 3. Supplementary Methods

5'Race Assay. The 5' RACE assay for both KIF11 and VEGF-A is shown below.



- Isolation of poly-A mRNA using poly-T
- Ligation of RNA oligo to 5' end of mRNA; reverse transcription with gene-specific cDNA primer
- First round amplification with adapter-specific forward primer and gene-specific reverse primer
- Second round amplification with nested adapter-specific forward primer and nested gene-specific reverse primer; scan for PCR product on Bioanalyzer
- Next Gen Sequencing of 2nd round PCR product; primer within adapter sequence
- Align Illumina sequence downstream of adapter to target transcript
- Calculate fraction of total aligned reads by cleavage position

Analysis of the 5' RACE Illumina data from KIF11 and VEGF-A was performed with a custom Perl program. Each Illumina read was first tested for the presence of a perfect adapter sequence and at least 24 bases downstream from the adapter ligation site. Counts of unique 24 base reads from each Illumina run were determined and stored in MySQL database. Each unique 24 base sequence was subsequently aligned to the target transcript using Bowtie (version 0.12.5; http://bowtie-bio.source-forge.net), with a maximum of 3 mismatches allowed for each alignment (bowtie argument "-v 3"). The percent of the total number of bowtie-aligned sequences was plotted as a function of position in the target transcript. The predicted cleavage site in either the VEGF-A or KIF11 transcript, based on the sequence of their respective siRNAs, as well as the resulting cleavage product for each is shown below:



P-values were determined using a student's t-test for percent of total reads at the expected cleavage site in post-dose versus basal or pre-dose samples. Statistics and histograms were generated in R.

DCE-MRI Methodology. Appropriate patients with hepatic and/or an assessable extrahepatic tumor (e.g., adrenal, retroperitoneal, bone) of ≥2 cm were serially imaged by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). For this purpose, each patient underwent 3 MRI examinations performed at baseline (within 14 days before the first dose in Cycle 1), at 48 to 96 hours following the first dose in Cycle 1, and at 7 to 9 days following the first dose in Cycle 1.
The MRI data was centralized, quality controlled and processed for quantitative analyses by BioClinica Inc. (Newtown, PA). Eight clinical sites were qualified to perform image acquisition in this study. The site qualification process included phantom scans (Eurospin, Test Object n°5) to check for the accuracy and consistency of T1 measurements. Each MRI examination included morphological pre-contrast MRI sequences (axial T1 and T2) covering the entire liver to facilitate the localization of tumors of interest. For DCE-MRI, three-dimensional spoiled Gradient Echo T1 sequences with variable Flip Angles (2, 5, 10, 15, 20, 25 and 30°) for T1 measurement were used. The DCE-MRI data were acquired in an oblique coronal orientation. Torso/abdominal phased array receiver coils were used. The injection of the contrast agent started one minute after the initiation of data acquisition and the total duration of the perfusion sequence was 9 minutes. The contrast agent dose was 0.1 mmol/kg, injected at a rate of 3 ml/sec and followed by a 20 ml saline flush.

The quantitative MRI parameters extracted from the DCE-MRI datasets included the transfer constant (Ktrans), the volume of Extra vascular Extracellular Space - EES (Ve) and the Initial Area Under Gadolinium Curve (IAUGC). Arterial Input Function (AIF) was semi-automatically extracted using a region of interest in the aorta. The AIF and tumor data were fit to gamma-variate curves. The bi-compartmental Tofts[1] model was used to describe the exchange between the plasma space and the EES and compute the perfusion parameters.

The localization and delineation of the tumors were performed by a blinded radiologist. Up to 3 tumors were selected for quantitative analyses. The tumors were manually delineated at each timepoint. The perfusion parameters were averaged across the volumes of interest representing the tumors.

Reference

1. Tofts, P.S., *et al*. Estimating kinetic parameters from dynamic contrast-enhanced T(1)-weighted MRI of a diffusible tracer: standardized quantities and symbols. *J Magn Reson Imaging*. **10**, 223-232 (1999).

Item 4. Supplementary Figure Legends

Supplementary Figure 1. ALN-VSP siRNA duplex designs. Shown are sequences and chemical modifications for KSP and VEGF siRNAs.

Supplementary Figure 2. Activity of ALN-VSP in murine orthotopic Hep3B liver tumor model. (a) Evidence of RNAi in tumors by 5' RACE assay in mice 24 hours after a single dose of 4 mg/kg ALN-VSP. Control consists of identical dose of LNP-luciferase (same LNP as ALN-VSP containing a siRNA targeting non-mammalian luciferase). Results of sequencing shown in top panels, with start position of each fragment shown on the x-axis and percent of total reads comprised by each fragment on the y-axis. The predicted specific VEGF or KSP mRNA cleavage product is indicated by the cyan bar; other fragments are shown in orange. Agilent microfluidic bioanalyzer DNA 1000 results from VEGF and KSP 5' RACE second round PCR shown in bottom panel, with arrows pointing to predicted VEGF (230 bp) and KSP (211 bp) mRNA cleavage products. (b) Unipolar mitotic spindles (indicated by white arrows) in tumors of animals treated with ALN-VSP. (c) Effects of VEGF targeting with either LNP-VEGF (6 mg/kg IV twice-weekly x 3 wks) or bevacizumab (5 mg/kg IP twice-weekly x 3 wks) on tumor hemorrhage and microvessel density 72 hours after last dose. Values are mean ±SD. *P-values compared to control derived from Student's T-test. (d) Survival curves of mice treated with 6 doses (bi-weekly x 3 weeks) of ALN-VSP or control. N=16 per group.

Supplementary Figure 3. Activity of ALN-VSP in murine Hep3B intraperitoneal tumor model. SCID/beige mice were injected intraperitoneally with 1 x 10^6 human Hep3B hepatocellular carcinoma cells. Fourteen days post-seeding, mice were given a single IV bolus injection of ALN-VSP or Control and sacrificed after 24 hours. (A) Tumor cell monoasters (indicated by white arrows) in tumors of animals treated with ALN-VSP (upper panel) but not in animals treated with Control (lower panel). (B) Quantitation of tumor cell monoasters in individual animals treated with ALN-VSP (1-4) or Control (5-8). ROI=Region of Interest.

Supplementary Figure 4. Study design for Phase I trial of ALN-VSP. W: week; d: day post-dose (dosing on day 1);Rx: ALN-VSP dosing; DCE-MRI: dynamic contrast-enhanced MRI. | indicates timepoint where DCE-MRI, tumor biopsy or CT scan was performed.

Supplementary Figure 5. Comparison of VEGF and KSP mRNA expression in liver and liver tumors. mRNA levelswere measured in banked tissue specimens (CRC: colorectal cancer liver metastasis, HCC: hepatocellular carcinoma, Liver: normal liver) or the Hep3B human hepatocellular carcinoma cell line by qPCR. Cp values were obtained and converted into molecules/μg of RNA based on a standard curve.

Supplementary Figure 6. Comparison of ALN-VSP PK in non-human primates (NHPs) and patients. Mean plasma PK results and standard deviations for KSP (left panel) and VEGF (right panel) siRNAs shown for NHPs (N=6) and for patients on the Phase I trial (N=4) following a 1 mg/kg dose of ALN-VSP.