Antitumor and Antimetastatic Activity of Ribozymes Targeting the Messenger RNA of Vascular Endothelial Growth Factor Receptors


ABSTRACT

Chemically stabilized hammerhead ribozymes are nuclease-resistant, RNA-based oligonucleotides that selectively bind and cleave specific target RNAs. Due to their potential for specifically inhibiting gene expression, ribozymes are being investigated for therapeutic applications as well as for the elucidation of gene function. In particular, we have investigated ribozymes that target the mRNA of the vascular endothelial growth factor (VEGF) receptors because VEGF signaling is an important mediator of tumor angiogenesis and metastasis. Here we report pharmacodynamic studies testing anti-Flt-1 (VEGFR-1) and anti-KDR (VEGFR-2) ribozymes in animal models of solid tumor growth and metastasis. Ribozymes targeting either Flt-1 or KDR significantly inhibited primary tumor growth in a highly metastatic variant of Lewis lung carcinoma. However, only treatment with the anti-Flt-1 ribozyme resulted in a statistically significant and dose-dependent inhibition of lung metastasis in this model. The anti-Flt-1 ribozyme was then tested in a xenograft model of human metastatic colorectal cancer in which significant inhibition of liver metastasis was observed. Taken together, these data represent the first demonstration that synthetic ribozymes targeting VEGF receptor mRNA reduced the growth and metastasis of solid tumors in vivo.

INTRODUCTION

In the early 1970s (1), Folkman hypothesized that solid tumor growth and metastasis are critically dependent on angiogenesis, the formation of new blood vessels from preexisting vasculature. Over the past few decades, many mediators of angiogenesis have been characterized, providing new and important targets for drug discovery research. Considerable effort has been directed toward the development of pharmacological agents that modulate specific pathways associated with angiogenesis.

Among the many known triggers of tumor angiogenesis, VEGF has emerged as a relatively specific effector (2, 3). In fact, VEGF expression has been observed in many human tumor types (4–10), is up-regulated in response to hypoxia (11, 12), and has been specifically linked with tumor neoangiulation (13–15). Tumor cells engineered to express VEGF constitutively exhibit enhanced tumor growth and angiogenic phenotypes (16). Conversely, treatments with anti-VEGF monoclonal antibodies have been shown to inhibit the growth of a variety of solid tumors in murine models (8, 17, 18).

Inhibition of VEGF signaling at the receptor level has been proposed as a means of inhibiting VEGF-dependent tumor growth and metastasis (19–21). The mitogenic selectivity of VEGF for endothelial cells is due to the relatively exclusive distribution of VEGF receptors in this cell type (2, 22). Two major human VEGF receptor subtypes have been identified and are known as Flt-1 (VEGFR-1) and KDR (VEGFR-2 or Flk-1 in the mouse) (23). Although Flt-1 and KDR/Flk-1 receptors both appear to be involved in angiogenesis, their respective roles in this process are only partially elucidated.

Both Flt-1 and KDR/Flk-1 receptors may play important roles in VEGF-stimulated cell proliferation. For example, the expression of the murine Flk-1 receptor is associated with proliferating endothelial cells (24), and human endothelial cells expressing the KDR receptor exhibit shape changes and enhanced DNA synthesis on stimulation with VEGF (25). Human umbilical vein endothelial cells expressing Flt-1 receptor mutants are resistant to VEGF-stimulated proliferation (26). In addition, Barleon et al. (21) demonstrated that purified VEGF and conditioned media from hypoxic tumor cell lines can up-regulate the expression of Flt-1 but not KDR/Flk-1 receptors in cultured endothelial cells.

Developmental models provide useful insight into the physiological roles of these receptors in vivo. Although mutations in either Flk-1 or Flt-1 are fatal to developing mouse...
embryos, their respective embryological abnormalities in vasculogenesis and angiogenesis differ (27–29). Abnormal Flk-1 expression leads to defective endothelial cell development (28, 29), whereas mutant Flt-1 embryos show normally differentiated endothelial cells that form highly abnormal and disorganized vascular structures (27). This body of evidence suggests that inhibition of VEGF receptor signaling could be an important target for therapeutic intervention in pathological angiogenesis.

Hammerhead ribozymes are trans-acting, RNA-based enzymes that specifically bind and cleave target RNA. In recent years, considerable effort has been directed toward the application of ribozyme technologies to inhibit specific gene expression (30). Stabilization chemistries have been developed to render these molecules highly resistant to serum nucleases (31) and hence suitable for in vivo applications. As a result, local delivery of chemically stabilized ribozymes has been reported to inhibit specifically cytokine-stimulated expression of stromelysin mRNA in a rabbit knee osteoarthrosis model (32) and VEGF-stimulated angiogenesis in a rat corneal pocket model (33). In addition, a stabilized ribozyme targeting protein kinase C α mRNA was shown to reduce sc implanted glioma tumor growth in rats (34).

We reported previously on ribozymes that target specific sites in the mRNA of each of the two major VEGF receptor subtypes, Flt-1 and KDR (33). Testing in cultured human microvascular endothelial cells was used as a way to choose the most effective anti-Flt-1 and KDR ribozymes to advance into animal studies. These cell culture studies resulted in the choice of one lead ribozyme site in each mRNA target. In particular, ribozymes targeting Flt-1 site 4229 or KDR site 726 were chosen from a panel of ribozymes targeting different sites spanning the length of the target mRNAs. These choices were based on the ability of the ribozyme to inhibit VEGF-stimulated cell proliferation and to decrease specifically their targeted mRNA in cell culture relative to attenuated controls. In subsequent in vivo studies, anti-Flt-1 and KDR ribozymes targeting these specific sites significantly decreased angiogenesis when tested in a corneal pocket model of VEGF-induced angiogenesis. In these two assays, controls that had the same binding arms as the ribozymes but reduced cleavage activity due to nucleotide changes in the catalytic core showed little, if any, effect. Here we report pharmacodynamic studies using systemic delivery of stabilized hammerhead ribozymes designed to inhibit the expression of the human VEGF receptors, Flt-1 or KDR. Each ribozyme is targeted to a conserved sequence in either Flt-1 or KDR mRNA that is also present in the mouse and rat, enabling rodent studies to be conducted with the same agent in these species. The Lewis lung carcinoma model was chosen for initial study because neovascularization in this tumor type is VEGF dependent (35, 36), and the highly metastatic line can be used to study the process of metastasis without resection of the primary tumor. In this model, both the anti-Flt-1 and the anti-KDR ribozyme inhibited primary tumor growth when administered iv by continuous infusion. However, only treatment with the anti-Flt-1 ribozyme resulted in a statistically significant and dose-dependent inhibition of lung metastasis in this model. In further testing of the anti-Flt-1 ribozyme in a model of human colorectal metastasis, the number of liver metastases was also significantly reduced.

Materials and Methods

Ribozyme Synthesis

Hammerhead ribozymes and their corresponding attenuated controls were synthesized and purified as described previously (31, 32, 37). Sequences and the locations of modified nucleotides are given in Table 1. The five remaining unmodified ribonucleotides in the catalytic core are essential for cleavage activity (31). The four phosphorothioate linkages at the 5′-end and the 3′-3′ inverted deoxyabasic residue at the 3′-end provide additional resistance to exonucleases (31). The attenuated ribozyme controls maintain the binding arm sequence of the parent ribozyme and are thus still capable of binding the mRNA target. However, they have two nucleotide changes in the core sequence (Table 1) that substantially reduce their ability to carry out the cleavage reaction (Fig. 1). Ribozymes were designed to target Flt-1 or KDR/Flk-1 mRNA sites conserved in human, mouse, and rat.

In Vitro Ribozyme Cleavage Assays

RNA cleavage rates were measured for the ribozymes and their paired attenuated controls using a 15-nucleotide synthetic complementary RNA substrate as described previously (38). Briefly, the ribozymes (500 nM) were incubated with stoichiometric amounts of substrate (and trace amounts of 32P-5′-end-labeled substrate) in 50 mM Tris-HCl buffer (pH 8.0) and 40 mM magnesium
MgCl$_2$ at 37°C. The magnesium-dependent cleavage reactions were quenched with EDTA at specified time points and assayed by PAGE with band quantitation using a Molecular Dynamics PhosphorImager (model 425E).

**Animal Experimentation**

All animal experimentation was performed in accordance with the Guide for the Care and Use of Laboratory Animals (39) and the policies and procedures of the Ribozyme Pharmaceuticals, Inc. and Chiron Corp. animal care and use programs.

**LLC-HM**

**Tumor Inoculations.** Female 6–8-week-old C57BL/6 mice (Harlan Bioproducts, Indianapolis, IN) were housed in groups of four and inoculated sc in the left flank with $5 \times 10^5$ LLC-HM cells from brei preparations of tumors exhibiting a highly metastatic phenotype in mice (six passages; the original LLC-HM cell line was kindly provided by Dr. Michael O’Reilly; Children’s Hospital and Harvard Medical School, Boston, MA).

**Ribozyme Administrations.** Three days after tumor inoculation, animals were anesthetized with 125 mg/kg ketamine and 8 mg/kg xylazine in sterile veterinary saline and placed in a supine position on a 37°C warming pad. The left jugular vein was exposed, and sc fat and fascia were cleared from the vessel. After tying off the jugular vein distally, a small incision was made in the jugular vein, and a catheter constructed from PE 50 polyethylene medical tubing (prefilled with sterile saline) was introduced into the vessel to approximately 0.5 cm. The vessel was secured to the catheter proximal to the incision with three ligatures and anchored to the surrounding muscle tissue using 4-0 silk. The catheter was fed through a trochar implanted sc, passing from the neck wound dorsally toward the nuchal region. When the catheter was in place and its volume had been replaced with test solution, it was attached to an Alzet osmotic minipump (flow rate, 12 ml/day). Pumps were filled with either ribozyme, attenuated control, or saline solutions and implanted sc through a dorsal incision to a final position caudal to the scapulae. Animals were allowed to recover on 37°C warming pads.

Test substances were dissolved in saline at concentrations ranging from 1.7–167 mg/ml. Beginning 3 days after tumor inoculation, animals were dosed at 0, 1, 3, 10, 30, and 100 mg/kg/day for 14 days. These doses correspond to 0, 3, 9, 30, 90, and 300 mg/m$^2$/day. Ten animals were used for each treatment group. Less than 10% of the 210 animals in the LLC-HM study were removed early, primarily because of catheter failures (i.e., detaching from the vessel or pump), or because the animal did not survive pump implantation. Thus, 7–10 animals remained in each group at the end of the study.

**Measurement of Tumor Growth and Metastasis.** In pilot experiments, LLC-HM-injected animals began to become moribund after about 24 days due to substantial lung tumor burden. To assess both primary tumor growth and metastatic disease in the same animal, the experiment was terminated on day 25, before control animals became moribund. Primary tumor length and width were measured with microlimiters every other day from postinoculum days 4–24. Tumor volume was

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**Fig. 1 In vitro ribozyme cleavage analysis.** A time course of *in vitro* cleavage analysis on a complementary synthetic RNA substrate was carried out for the ribozymes and paired attenuated controls targeting (A) Flt-1 site 4229 and (B) KDR site 726. The upper and lower bands on the polyacrylamide gel correspond to full-length and cleaved RNA substrate, respectively. Along with stoichiometric amounts of unlabelled RNA substrate, a trace amount of $^{32}$P-5’-end-labeled RNA substrate was included in the reactions for detection purposes. Based on PAGE analysis of the time course of cleavage, ribozyme cleavage rates were calculated using a double exponential curve fit as described previously (38). According to the data, the first-order rate constants ($k_1$) for the anti-Flt-1 ribozyme and control were $0.464 \pm 0.005$ min$^{-1}$ and $0.001 \pm 4 \times 10^{-5}$ min$^{-1}$, respectively. The corresponding $k_1$ values for the anti-KDR ribozyme and control were $0.780 \pm 0.071$ min$^{-1}$ and $0.0002 \pm 2 \times 10^{-4}$ min$^{-1}$, respectively.
calculated using the following formula: \(0.5\text{length}(\text{width}^2)\). Twenty-five days after inoculation, animals were killed, and tumors and lungs were removed. The numbers of surface macrometastases on the lung were counted under a dissecting microscope at a magnification of \(\times 25\).

Data Analysis. Group tumor volume means from each treatment group obtained from days 12–24 postinoculation were subjected separately to normality testing and one-way ANOVA. Treatment group means for ribozymes and their paired attenuated controls were compared using a Tukey-Kramer post hoc test for significance (\(\alpha = 0.05\)). The mean numbers of pulmonary metastases from each treatment group (including the saline control) were subjected to normality testing and one-way ANOVA, followed by a Dunnett’s test for significance (\(\alpha = 0.05\)) using the saline group as the control.

**KM12L4a Human Colorectal Carcinoma/Liver Metastasis Model**

**Tumor Inoculations.** Male 6–8-week-old nude mice (Charles River Laboratories) were housed in groups of five. On day 0, each spleen was inoculated with \(5 \times 10^5\) KM12L4a cells within the splenic capsule as described below. KM12L4a cells were obtained from Dr. Jerald Killion (University of Texas M. D. Anderson Cancer Center, Houston, TX). Cells were grown in DMEM with 2 mM glutamine, 10% FBS with nonessential amino acids, sodium pyruvate, and vitamins. On day 5 after tumor cell inoculation, the spleens were removed from the animals.

The tumor cell inoculation was performed as follows: (a) animals were anesthetized with 125 mg/kg ketamine and 8 mg/kg xylazine; (b) an abdominal incision of \(\sim 3\) mm was made to the left of the midline; and (c) the spleen was gently exteriorized. The KM12L4a cell suspension was in inoculated into the spleen after slowly inserting a 28-gauge needle into the large pole of the spleen until the bevel of the syringe became visible in the central portion of the spleen just beneath the splenic capsule.

**Ribozyme Administrations.** Three days after inoculation, animals were surgically implanted with sc 14-day Alzet osmotic minipumps (flow rate, 12 \(\mu\)L/day) filled with either saline or anti-Flt-1 ribozyme solutions. Animals were dosed at 0, 12, 36, and 100 mg/kg/day for 14 days. These administered doses correspond to 0, 36, 108, and 300 mg/m\(^2\)/day. On day 18, the initial pumps were removed and replaced with fresh minipumps containing either saline or the same doses of anti-Flt-1 ribozyme. Animals were observed daily after each surgery, and healing of the surgical incision sites was unaffected by any treatment as compared with the saline control group. The study was ended on day 41.

Fifteen animals were included in each treatment group. If tumor cells successfully seed the liver, metastatic tumor formation in this xenograft model is usually rapid and aggressive; however, a poor tumor take is possible. Animals were randomly distributed before assignment to treatment groups to address the variable outgrowth properties of this model. Several animals were removed from the study as follows: (a) saline group, one animal was killed on day 33 because of the tumor burden; (b) 12 mg/kg/day group, one animal was killed early (day 17) because of continued loss of body weight, and one animal was found dead on day 35; (c) 100 mg/kg/day, one animal did not survive cell implantation, and one animal did not survive pump replacement. Thus, the final sample size for the saline-treated group and the 12, 36, and 100 mg/kg/day dose groups was 14, 13, 15, and 13 mice, respectively.

![Fig. 2](Image) Inhibition of LLC-HM primary tumor growth (day 18). Primary tumor volume measured at day 18 is given for LLC-HM tumor-bearing animals treated with (A) the anti-Flt-1 ribozyme (■), or the anti-Flt-1 attenuated control (□) or (B) the anti-KDR ribozyme (●) or the anti-KDR attenuated control (○). The dose range is 1–100 mg/kg/day. For reference, the mean tumor volume ± SEM for the saline-treated group (\(n = 10\) animals) is indicated by the gray bar in both panels. Animals received continuous iv administration of ribozyme or attenuated control for 14 days, beginning on day 3 after tumor inoculation. Thus, these day 18 data represent tumor volumes as measured 1 day after the cessation of treatment. Data for each group (\(n = 7–10\) animals) are presented as mean tumor volume ± SEM. * \(P < 0.05\) versus saline (Dunnett’s test for significance); † \(P < 0.05\) for active ribozymes compared to their time- and dose-matched paired attenuated controls (Tukey-Kramer post hoc test for significance). Where statistically significant, the percentage of inhibition of tumor growth versus saline is indicated on the graph. Direct comparisons to the attenuated controls are as follows: for the anti-Flt-1 ribozyme (A), doses of 10 and 30 mg/kg/day resulted in a statistically significant 63% and 89% inhibition of tumor growth relative to the attenuated control, respectively. For the anti-KDR ribozyme (B), only the highest dose (100 mg/kg/day) resulted in a statistically significant inhibition of tumor growth relative to its paired attenuated control (72% inhibition).
Measurement of Liver Weight and Liver Metastasis. On day 41, all surviving animals were killed by CO₂ inhalation. Livers were removed, weighed, and scored for liver metastases. In cases where the number of tumor foci was too numerous to count (i.e., >100), a count of 101 was applied.

Data Analysis. All statistical testing was performed at the α = 0.05 level. Because the individual numbers of liver metastases in each treatment group were not normally distributed, group means were compared using the Kruskal-Wallis nonparametric rank test at 95% confidence limits. Individual liver weight means were normally distributed. The liver weight data were subjected to ANOVA followed by Dunnett’s test for significance. Differences between treatment groups with respect to the presence or absence of metastases were compared using the χ² test.

RESULTS

Catalytic Cleavage Rates of Ribozymes and Paired Attenuated Controls. In previous in vivo studies, anti-Flt-1 and KDR ribozymes significantly decreased angiogenesis when tested in a corneal pocket model of VEGF-induced angiogenesis (33). Attenuated controls were included that retained the binding arm sequence of the parent ribozymes and hence were still capable of binding to their intended mRNA target. However, these controls had two nucleotide changes in the core region (sequences shown in Table 1) that substantially reduced their ability to carry out the cleavage reaction. These controls had negligible effect on the end points measured previously in cell culture and only reduced angiogenesis at the highest doses tested in the corneal model. Thus, for direct comparison, similar controls were included in the initial Lewis lung carcinoma studies reported here.

Although these initial in vivo data were established using 3-bp stem II ribozymes, the 4-bp stem II versions (Table 1) were used in the current studies. This decision was based, in part, on the higher in vitro cleavage activity of the 4-bp stem II ribozymes compared with that of the 3-bp stem II ribozymes (see below), although their activities in cell culture (33) and in the corneal pocket model are comparable (data not shown).

Fig. 1 shows the results of in vitro catalytic cleavage assays conducted for the 4-bp stem II anti-Flt-1 and anti-KDR ribozymes, along with their paired attenuated controls. The changes in the core resulted in attenuated analogues that have in vitro cleavage rates that are at least 2 orders of magnitude lower than those of the active ribozymes. For the anti-Flt-1 ribozyme and its paired attenuated control, the first-order rate constants (k1) were 0.464 ± 0.005 min⁻¹ and 0.001 ± 4 × 10⁻⁵ min⁻¹, respectively. For the anti-KDR ribozyme and its paired control, k1 values were 0.780 ± 0.071 min⁻¹ and 0.0002 ± 2 × 10⁻⁴ min⁻¹, respectively. For comparison, k1 values for the 3-bp stem II versions of these ribozymes reported previously in the corneal model study (33) were lower (0.081 ± 0.007 and 0.434 ± 0.024 min⁻¹ for the anti-Flt-1 and anti-KDR ribozyme, respectively). These cleavage data, together with the original cell culture and corneal model results (33), demonstrate that the effect observed for active ribozymes occurs through a ribozyme-specific (i.e., cleavage) mechanism.

Effect of Anti-Flt-1 or KDR Ribozymes on LLC-HM Primary Tumor Growth. Neovascularization of Lewis lung carcinoma tumors depends on VEGF (35, 36). A highly metastatic variant of Lewis lung carcinoma (LLC-HM) was selected in the present study because it allows for the assessment of primary tumor growth and metastasis without resection of the primary tumor. Thus, both the antitumor and antimetastatic effects of the anti-Flt-1 and anti-KDR ribozymes (together with their respective attenuated controls) were evaluated in the LLC-HM model after continuous iv administration. Pilot studies conducted before the current study clearly demonstrated that the anti-Flt-1 or the anti-KDR ribozyme did not affect the healing of
any surgical wounds associated with jugular catheterization and/or implantation of the Alzet minipump in mice (data not shown).

In general, both anti-Flt-1 and anti-KDR ribozymes inhibited primary tumor growth within the 1–100 mg/kg/day iv dose range. The effect on primary tumor growth observed immediately after the dosing period (day 18) is given in Fig. 2. The anti-Flt-1 ribozyme (Fig. 2A) reduced LLC-HM primary tumor growth in a dose-dependent manner as compared with either saline or its corresponding attenuated control. At the lowest dose (1 mg/kg/day), both the anti-Flt-1 ribozyme and its attenuated control reduced primary tumor growth similarly versus saline controls (~50%; P < 0.001 for both observations). However, with increasing dose, active anti-Flt-1 ribozyme reduced primary tumor growth to a greater extent than the attenuated control (P < 0.001 for 10 and 30 mg/kg/day dose groups). The attenuated control showed no further tumor growth inhibition until the highest dose (100 mg/kg/day). The greatest inhibition of tumor growth by the active anti-Flt-1 ribozyme (92% as compared with saline; P = 0.0001) and the largest significant difference between active Flt-1 ribozyme and its attenuated control (89%; P = 0.0001) occurred at a dose of 30 mg/kg/day. Mean tumor volumes for the active anti-Flt-1 ribozyme and attenuated control groups at this dose were 65.34 ± 37.18 and 585.45 ± 98.67 mm³, respectively, compared with 830.86 ± 82.53 mm³ for the saline control group.

The differences between the antitumor effects of the anti-KDR ribozyme and its corresponding attenuated control were more complex (Fig. 2B). At a dose of 1 mg/kg/day, the anti-KDR ribozyme had no effect on primary tumor growth versus the saline control. However, at all higher doses (3–100 mg/kg/day), the anti-KDR ribozyme significantly inhibited primary tumor growth as compared with the saline control by about the same degree (68–75%; P < 0.0001 for all observations). The antitumor dose relationship profile of the attenuated control was more complicated. Doses of 1 and 3 mg/kg/day showed significant tumor growth inhibition compared with saline (54–57%; P = 0.0019 for both observations). In contrast, higher doses of the attenuated control had no effect on tumor growth. For the anti-KDR ribozyme, 10 mg/kg/day was the lowest dose that resulted in significant inhibition of primary tumor volume (68%; P = 0.0001) where the corresponding attenuated control had no significant effect (versus saline). A statistically significant difference between the effects of the active anti-KDR ribozyme and its attenuated control was detected at the 100 mg/kg/day dose (72%; P < 0.001).

A more complete view of the integrated difference between the active ribozymes versus their attenuated controls is provided by the primary tumor growth characteristics from the entire 24-day growth period. Overall, the greatest difference between the ribozymes and their controls, even after dosing was completed, was observed with a dose of 30 mg/kg/day for the anti-Flt-1 ribozyme and a dose of 10 mg/kg/day for the anti-KDR ribozyme. Primary tumor growth curves for these two treatments are shown in Fig. 3. For the anti-Flt-1 ribozyme (Fig. 3A), the maximum tumor volume reduction compared with the saline control was 92% immediately after the last day of dosing (day 18) and 77% at day 24. Moreover, the maximum tumor volume reduction for the anti-Flt-1 ribozyme compared with its attenuated control was 89% on day 18 and 65% at day 24. It should be noted that the reduction observed with the anti-Flt-1 ribozyme from day 18 through day 24 was significant compared with both the saline and attenuated controls, even though ribozyme treatment was discontinued on day 17 (P < 0.05).

With 10 mg/kg/day anti-KDR ribozyme (Fig. 3B), the maximal tumor volume reduction compared with saline was 68% on day 18 (P = 0.0001) and 60% on day 24 (P < 0.05). In fact, the reduction in tumor growth at this dose was statistically significant when compared with saline from days 12–24 (P < 0.05 for all observations). In contrast, LLC-HM tumor growth was not significantly reduced by treatment with the anti-KDR attenuated control at this dose, except as noted on day 22. Even

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**Fig. 4 Reduction of LLC-HM pulmonary metastasis.** Pulmonary macrometastases were counted on day 25 after tumor inoculation and averaged for each treatment group (n = 7–10 animals). Data are reported as the mean number of metastases ± SEM over the dosing range of 1–100 mg/kg/day. Shown are data after treatment with (A) the anti-Flt-1 ribozyme [■] or its paired attenuated control [□] or (B) the anti-KDR ribozyme [●] or its paired attenuated control [○]. The mean number of metastases ± SEM for the saline group is indicated by the gray bar in both panels. *P < 0.05 for comparison to saline (Dunnett’s test for significance). Where statistically significant, the percentage reduction in metastases versus saline treatment is indicated on the graph.
Inhibition of colorectal cancer metastasis to the liver by the anti-Flt-1 ribozyme. Metastases to the liver 41 days after splenic inoculation of KM12L4a cells are reported. Data are reported as the number of metastases in individual mice in each treatment group (\( n \)) and as the mean number of metastases \( \pm \) SEM (\( n \)).

### Table 2: Results of anti-Flt-1 ribozyme treatment in the KM12L4a colorectal cancer model of liver metastasis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver metastatic incidence</th>
<th>Liver weight*</th>
<th>Liver metastases</th>
<th>Liver metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>12/14</td>
<td>59 ( \pm ) 13</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>12 mg/kg/day</td>
<td>11/13</td>
<td>3.1 ( \pm ) 0.6</td>
<td>63 ( \pm ) 14</td>
<td>101</td>
</tr>
<tr>
<td>36 mg/kg/day</td>
<td>13/15</td>
<td>2.2 ( \pm ) 0.3</td>
<td>30 ( \pm ) 12 (( P = 0.03 ))</td>
<td>5 (( P = 0.03 ))</td>
</tr>
<tr>
<td>100 mg/kg/day</td>
<td>6/13 (( P = 0.03 ))</td>
<td>1.8 ( \pm ) 0.3</td>
<td>17 ( \pm ) 10 (( P = 0.03 ))</td>
<td>0 (( P = 0.03 ))</td>
</tr>
<tr>
<td>Observation group</td>
<td>NA</td>
<td>2.0 ( \pm ) 0.1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

- * Some livers had large multilobular tumor clusters consisting of numerous (>100) tumor foci that occupied from 30–80% of the liver lobes of some animals. These animals were scored as having >100 tumor foci/liver, and a value of 101 liver metastases was entered for statistical analysis.
- * Liver metastatic incidence = the number of livers with one or more metastases/total number of livers in the group. Differences were assessed by \( \chi^2 \) methodology.
- * Liver weights were normally distributed (ANOVA) and are reported as mean \( \pm \) SEM. There were no statistically significant differences in liver weights between saline- and ribozyme-treated groups based on Dunnett’s test for significance.
- * Liver metastases were counted, and \( P \)s were calculated based on comparison to saline using a nonparametric rank test (Kruskal-Wallis).
- * NA, not applicable because animals were not inoculated with tumor cells.

so, the differences in tumor volume reduction between the active anti-KDR ribozyme and its attenuated control were statistically different only on a limited number of days, e.g., day 20 where a 56% difference was noted (\( P < 0.05 \)).

### Antimetastatic Activity of Anti-Flt-1 and KDR Ribozymes in LLC-HM Tumor-bearing Mice

The antimetastatic activities of the anti-Flt-1 and anti-KDR ribozymes and their attenuated controls were also determined (Fig. 4). For this assessment, lung macrometastases were counted at the end of the study (postinoculation day 25 and 8 days after the cessation of treatment). As shown in Fig. 4A, the anti-Flt-1 ribozyme reduced the number of pulmonary metastases with respect to saline controls in a dose-dependent manner. The greatest antimetastatic activity with respect to saline controls (78% and 83% inhibition) was observed at doses of 30 and 100 mg/kg/day anti-Flt-1 ribozyme, respectively (\( P = 0.005 \) for both observations). In contrast, no antimetastatic activity was observed with the anti-Flt-1 attenuated control; the data were statistically indistinguishable from saline controls at all doses and showed no dose-dependent trend.

Treatment with the anti-KDR ribozyme also reduced the number of pulmonary metastases (Fig. 4B), but not to the same degree as the anti-Flt-1 ribozyme. For the dose range of 3–100 mg/kg/day, the anti-KDR ribozyme reduced the number of metastases \( \sim 50–60\% \), although this was not significantly different compared with the saline control group. Interestingly, any reduction in the number of pulmonary metastases present at the lowest doses of the attenuated control was not observed at the higher doses. No dose of the anti-KDR attenuated control resulted in a statistical reduction of lung metastases (versus saline control).

### Inhibition of Liver Metastasis in a Model of Human Colorectal Cancer

Because the anti-Flt-1 ribozyme exhibited a slightly more robust inhibition of primary tumor growth and also significantly inhibited lung metastasis in the LLC-HM model, this ribozyme was tested further in a metastatic model of human colorectal cancer. In this model, human colorectal cancer cells (KM12L4a) were implanted into the spleen of mice, and after 5 days, the spleens were removed. Continuous sc administration of the anti-Flt-1 ribozyme was initiated 3 days after the splenic inoculation and continued for 28 days. At the end of the study (day 41), livers were weighed, and spontaneous metastases to the liver were counted. Although iv dosing was used in the LLC-HM study reported above, subsequent pharmacokinetic studies with the anti-Flt-1 ribozyme in mice also supported sc dosing. The anti-Flt-1 ribozyme is \( \sim 80\% \) bioavailable after sc administration, and its t\(_{1/2}\) in plasma after a single administration is similar to that seen with iv dosing (40). Thus, a sc route of administration was used in this xenograft model in an effort to avoid the surgical complications associated with chronic vascular catheterization in immunocompromised mice.

The effects on indices of liver metastasis of tumor-bearing animals after treatment with 12, 36, or 100 mg/kg/day anti-Flt-1 ribozyme are listed in Table 2 and are shown graphically in Fig. 5. As seen in Table 2, there was no statistical difference in liver weight between ribozyme-treated and control animals. However, there was a clear decrease in metastatic incidence at 100 mg/kg/day (\( P = 0.03 \)) as well as a dose-dependent reduction in the mean number of spontaneous metastases to the liver (\( P = \))
suggest a ribozyme-specific (cleavage) mechanism of action. Significant only at the higher doses, if at all. These results indicate attenuated controls. In addition, these controls behaved as expected in the corneal model of VEGF-induced angiogenesis in that their effect on decreasing angiogenesis was more potent than that observed in the colorectal cancer model. Based in part on these data, the anti-Flt-1 ribozyme on liver metastasis in a model of human colorectal cancer are described. Based in part on these data, the anti-Flt-1 ribozyme (ANGIOZYME) is currently being developed for clinical oncology indications.

In supporting cell culture studies (33), a significant decrease in cell proliferation was demonstrated with the active anti-Flt-1 and anti-KDR ribozymes, but not with their corresponding attenuated controls. In addition, these controls behaved as expected in the corneal model of VEGF-induced angiogenesis in that their effect on decreasing angiogenesis was significant only at the higher doses, if at all. These results suggest a ribozyme-specific (cleavage) mechanism of action.

In the pharmacodynamic studies reported here, the active anti-Flt-1 and KDR ribozymes exhibited antitumor activity. Treatment with the anti-Flt-1 ribozyme reduced LLC-HM primary tumor growth (Fig. 2A) and the number of lung metastases (Fig. 4A) in a dose-dependent manner. Moreover, the number of spontaneous liver metastases in the colorectal cancer model was significantly reduced after treatment with the two highest doses of the anti-Flt-1 ribozyme (Table 2; Figs. 5 and 6). For the anti-KDR ribozyme, all doses except the lowest dose significantly inhibited LLC-HM primary tumor growth (Fig. 2B). In stark contrast to the anti-Flt-1 ribozyme, there was no significant effect on lung metastasis at any dose of the anti-KDR ribozyme (Fig. 4B).

Whereas the anti-Flt-1 attenuated control had minimal effects on primary tumor growth and no effect on metastasis, some unexpected effects of the anti-KDR attenuated controls were noted on primary tumor growth, particularly at the lowest doses tested (Figs. 2B and 4B). For the anti-KDR attenuated control, the lower doses inhibited primary tumor growth, but the higher doses showed no inhibition. It is important to note that the anti-KDR control failed to inhibit cell proliferation in cultured human endothelial cells (33) and did not statistically inhibit angiogenesis in the VEGF-induced rat corneal model (data not shown). Additional studies would be necessary to characterize any specific role of this modified oligonucleotide in the complex processes involved in tumor growth.

The specific roles of each VEGF receptor in VEGF-induced endothelial cell proliferation and angiogenesis are poorly understood. It is clear that the KDR/Flk-1 receptor mediates VEGF-induced endothelial cell proliferation, shape, and motility changes (25, 41). KDR/Flk-1 is also involved in embryonic vasculogenesis and angiogenesis (24, 28, 29). However, evidence is now emerging for the participation of the Flt-1 receptor in endothelial cell proliferation and angiogenesis. Barleon et al. (21) demonstrated that VEGF stimulation of Flt-1 receptors in cultured endothelial cells enhances DNA synthesis. In addition, Fong et al. (27) demonstrated that Flt-1 receptor

**DISCUSSION**

VEGF has long been shown to play a major role in the recruitment and maintenance of blood supply for growing and metastasizing tumors (3). Recently, the inhibition of VEGF signaling at the receptor level has been proposed as a therapeutic strategy for the treatment of solid cancers (19, 20). The present study illustrates the antitumor and antimetastatic effects of chemically stabilized hammerhead ribozymes targeting either the Flt-1 or the KDR VEGF receptor mRNA in a metastatic tumor model in which neovascularization is thought to be dependent on VEGF expression (35, 36). In addition, the inhibitory effects of the anti-Flt-1 ribozyme on liver metastasis in a model of human colorectal cancer are described. Based in part on these data, the anti-Flt-1 ribozyme (ANGIOZYME) is currently being developed for clinical oncology indications.

In supporting cell culture studies (33), a significant decrease in cell proliferation was demonstrated with the active anti-Flt-1 and anti-KDR ribozymes, but not with their corresponding attenuated controls. In addition, these controls behaved as expected in the corneal model of VEGF-induced angiogenesis in that their effect on decreasing angiogenesis was significant only at the higher doses, if at all. These results suggest a ribozyme-specific (cleavage) mechanism of action.

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Anticancer Effects of Ribozymes Targeting Angiogenesis


It is clear that VEGF is involved in tumor growth and metastasis. Studies have shown that enhanced VEGF expression in tumors leads to increased tumor growth (16), whereas therapies that target VEGF itself reduce tumor growth and neovascularization as well as metastasis (8, 17, 18, 42). Thus, it is reasonable to assume that interfering with VEGF receptor function should also impact primary tumor growth and metastasis. Recently, the inhibition of VEGF receptor function has been demonstrated as an alternative means of cancer therapy. The expression of a tyrosine kinase-deficient Flk-1/KDR mutant resulted in reduced blastoma tumor growth and neovascularization in vivo (43). Strawn et al. (20) have shown that small molecule Flk-1/KDR antagonists reduce angiogenesis in a cho-roioallantoic membrane model. Several of these tyrosine kinase inhibitors are currently in clinical trials for a variety of clinical indications. Additionally, Shibuya et al. (19, 44) presented evidence that the Flt-1 receptor is involved in tumor angiogenesis. The results from the current study reinforce the hypothesis that targeting the VEGF/VEGF receptor system is a promising indication. Shibuya et al. (19) presented data suggesting that Flt-1 is a target for tumor growth inhibition. Cancer Res., 55: 5421–5425, 1995.

In addition to potential roles as therapeutics, ribozymes targeting VEGF receptor mRNA can also be used to determine the relative role(s) of each subtype in signal transduction and on downstream biological effects. The anti-Flt-1 and anti-KDR ribozymes provide unique tools for elucidating the functional roles of VEGF receptors in the angiogenesis, growth, and metastasis of VEGF-expressing tumors.

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