Recombinant Human Angiostatin by Twice-Daily Subcutaneous Injection in Advanced Cancer: A Pharmacokinetic and Long-Term Safety Study

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ABSTRACT

Purpose: A clinical study was performed to evaluate the pharmacokinetics (PK) and toxicity of three dose levels of the angiogenesis inhibitor recombinant human (rh) angiostatin when administered twice daily by s.c. injection.

Experimental Design: Eligible patients had cancer not amenable to standard treatments. Three groups of 8 patients received 7.5, 15, or 30 mg/m²/day divided in two s.c. injections for 28 consecutive days followed by a 7-day washout period. PK assessment was done at days 1 and 28. Thereafter, in absence of toxicity or a 100% increase in tumor size, treatment was continued without interruption.

Results: Median age was 53 years (range, 43–75), male:female ratio 10:14, Eastern Cooperative Oncology Group performance 0–1. At the range of doses evaluated, serum PK of all 24 of the patients showed linear relation between dose and area under the curve (AUC) and Cmax (reached after 2 h). Thirteen of 24 patients developed erythema at injection sites (11 patients, CTC grade 1; 2 patients, CTC grade 2) without pain or itching, spontaneously resolving within 2–3 weeks of treatment. Two patients went off study after developing hemorrhage in brain metastases, and 2 patients developed deep venous thrombosis. No other relevant treatment-related toxicities were seen, even during prolonged treatment. A panel of coagulation parameters was not influenced by rhAngiostatin treatment. Long-term (>6 months) stable disease (<25% growth of measurable uni- or bidimensional tumor size) was observed in 6 of 24 patients. Five patients received rhAngiostatin treatment for >1 year (overall median time on treatment 99 days).

Conclusions: Long-term twice-daily s.c. treatment with rhAngiostatin is well tolerated and feasible at the selected doses, and merits additional evaluation. Systemic exposure to rhAngiostatin is within the range of drug exposure that has biological activity in preclinical models.

INTRODUCTION

Development of angiostatin as a potential anticancer therapeutic agent originates from the observation that the removal of a s.c. implanted Lewis lung carcinoma resulted in enhanced growth of lung metastases. It was hypothesized that the presence of the primary tumor induced a circulating inhibitor of angiogenesis (1, 2). Subsequently, a protein with homology to the first four kringle domains of Plg was purified from the urine of Lewis lung tumor-bearing mice (3). Additional studies revealed that this fragment of Plg was an inhibitor of angiogenesis. Thereafter a 38 kDa proteolytic fragment of Plg consisting of the first four kringle domains became known as angiostatin. Treatment of mice with angiostatin inhibits outgrowth of tumors and their metastases (4). The combination of angiostatin with radiation therapy (5) or chemotherapy (6) enhances antitumor effect. Several proteins, such as ATP synthase (7, 8) and angiomotin (9), have been found to interact with angiostatin possibly modulating its antiangiogenic properties. Presently, the mechanism by which angiostatin exerts this antiangiogenic effect has not been elucidated (reviewed in Refs. 4, 10, 11).

A recombinant protein, rhAngiostatin, consisting of the first three kringle domains of Plg (12), was produced in Pichia Pastoris (13) for clinical development. In the first clinical study, rhAngiostatin (EntreMed) was given to cancer patients by single daily i.v. bolus dosing up to a dose of 240

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3 The abbreviations used are: Plg, plasminogen; rh, recombinant human; MAb, monoclonal antibody; AUC, area under the curve; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; DVT, deep venous thrombosis; BSA, body surface area; CT, computed tomography; Cmax, maximum concentration.
mg/m²/day. This treatment was safe, and the dose-limiting toxicity was not reached (14). In animal studies we showed that continuous exposure to angiostatin is more effective than bolus injection regarding inhibition of tumor growth (15). Because of loss of activity of the current formulation of rhAngiostatin at room temperature, a trial design incorporating continuous infusion is not feasible. Therefore, we designed a clinical trial evaluating rhAngiostatin administered as a bid (twice daily) schedule by s.c. injection, with the intention of mimicking the pharmacologic profile of continuous infusion.

Because rhAngiostatin differs one amino acid from the corresponding sequence in human Plg we hypothesized that s.c. injections may result in antibody formation. To monitor this carefully we included a number of 8 patients per cohort.

The primary goal of this study was to investigate the pharmacokinetic behavior and toxicity of the current formulation of rhAngiostatin when given in a bid schedule s.c. at the selected dose levels. Secondary end points were to study antibody generation, to evaluate tumor response rates, and to determine the effect of rhAngiostatin on plasma and urine levels of proangiogenic and coagulation factors.

MATERIALS AND METHODS

The protocol was approved by the ethics committee of the University Medical Center Utrecht (Utrecht, the Netherlands) according to local and national ethic and regulatory rules. Twenty-four patients with solid malignancies were entered in the study between December 2000 and May 2001. All of the patients gave voluntary, written informed consent. All of the patients were treated at the Department of Medical Oncology of the University Medical Center Utrecht.

Patients and Treatment Schedule

Histologically confirmed diagnosis of any solid malignant tumor not being amenable to standard therapy was required to include a patient in the study. Eligibility criteria additionally included a performance status of 0 or 1 (Eastern Cooperative Oncology Group), uni- or bidimensionally measurable tumor (by CT, magnetic resonance imaging, or physical examination), a life expectancy of >3 months, and age ≥18 years. Patients were ineligible in cases of clinical evidence of central nervous system involvement, recent major surgery, pregnancy, additional major medical or psychiatric illness, ongoing reversible side effects of prior therapy, active infection, or ongoing therapy with antiplatelet drugs or anticoagulants. Patients were also ineligible when their medical history included any of the following: myocardial infarction in the previous 6 months, uncontrolled angina pectoris, uncontrolled congestive heart failure, any malignancy other than the current cancer within 3 years, hypercoagulable condition, or active bleeding disorder. It was mandatory for all of the patients to use effective contraceptives during the study.

Laboratory values required for all of the patients were aspartate aminotransferase and alanine aminotransferase ≤1.2, and activated partial thromboplastin time ≤upper limit of normal range +2 s. Eight patients/dose level were treated, receiving 7.5, 15, or 30 mg/m²/day, respectively. Initial treatment schedule consisted of 28 days of bid injection, with a 12-h interval between injections, followed by a 7-day observation without treatment. This washout period allowed toxicity evaluation and the monitoring of potential withdrawal-evoked adverse events. For purposes of pharmacokinetic analyses, the second dose on day 1 was omitted. Starting from day 36, treatment was uninterrupted, in treatment cycles of 28 consecutive days. Patients were allowed to go off treatment for a maximum of 2 weeks after an uninterrupted treatment of at least 3 months.

The next dose level was opened after all of the patients in the previous level had completed the initial treatment schedule of 35 days without dose-limiting toxicities. Dose-limiting toxicity was defined as the occurrence of any treatment-related grade 4 hematological toxicity or grade 3 or greater nonhematological toxicity, using the National Cancer Institute Common Toxicity Criteria version 2.0.4

Toxicity evaluation (by assessing signs and symptoms in the out-patient clinic) was done every week during the first 5 weeks and thereafter once every 2 weeks. Tumor assessment was performed between days 28 and 35 from the start of treatment, and next every second month. Tumor response was assessed according to WHO criteria (16).

All of the patients underwent laboratory tests and physical examination at the day of screening, as well as at days 1, 15, 28, and 35, and thereafter once every 4 weeks. When clinically indicated, tests were repeated more frequently. Laboratory analyses included complete blood count of platelets, WBCs and differential, hemoglobin, urine analysis, uric acid, creatinin, total protein, albumin, bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, glucose, calcium, phosphorus, chloride, sodium, and potassium. A panel of coagulation parameters was determined from citrated plasma to assess whether blood coagulation changed during treatment with this drug. Measurements were done for international normalized ratio of prothrombin time, activated partial thromboplastin time, fibrin degradation products, tissue-type Plg activator, von Willebrand factor, Plg activator inhibitor-1, Plg, Plg α-2 antiplasminogen complex, thrombomodulin, tissue factor, and tissue factor pathway inhibitor.

Treatment was discontinued if patients experienced dose-limiting toxicity, reached >100% increase in the sum of unidimensional or the sum of the products of the bidimensional tumor sizes, or the appearance of tumor ≥1 cm at new sites, chose to withdraw, missed >14 consecutive days of treatment, pregnancy, or at the discretion of the investigator.

Drug and Dosage

rhAngiostatin is expressed in and purified from P. Pastoris, a methylotrophic yeast, and comprises the first three kringles normalized ratio of prothrombin time ≤1.2, and activated partial thromboplastin time ≤upper limit of normal range +2 s. Eight patients/dose level were treated, receiving 7.5, 15, or 30 mg/m²/day, respectively. Initial treatment schedule consisted of 28 days of bid injection, with a 12-h interval between injections, followed by a 7-day observation without treatment. This washout period allowed toxicity evaluation and the monitoring of potential withdrawal-evoked adverse events. For purposes of pharmacokinetic analyses, the second dose on day 1 was omitted. Starting from day 36, treatment was uninterrupted, in treatment cycles of 28 consecutive days. Patients were allowed to go off treatment for a maximum of 2 weeks after an uninterrupted treatment of at least 3 months.

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Drug and Dosage

rhAngiostatin is expressed in and purified from P. Pastoris, a methylotrophic yeast, and comprises the first three kringles

domains of Plg (amino acids 97–357), with a single amino acid mutation (N308 to E308) to prevent N-glycosylation. The recombinant human protein has been manufactured at large scales (13). The product was supplied as a sterile, clear, colorless to slightly pink solution without preservatives with a final concentration of 15 mg/ml in saline (produced by EntreMed). rhAngiostatin was stored at −70°C, thawed under sterile conditions, and drawn into 1-ml syringes containing the required dosage per patient in the hospital pharmacy. Syringes were then transported to the patient homes on dry ice, which ensured a temperature below −20°C. At the homes of the patients, syringes were transferred to a freezer, previously confirmed to maintain temperatures below −18°C (data not shown). Before injection, syringes were thawed for 30–45 min at room temperature and subsequently injected. Biological activity of the formulation of rhAngiostatin after initial thawing for syringe preparation was retained for at least 4 days. This was confirmed in an experimental tumor model (data not shown). Thereafter, twice-weekly prefilled syringes were supplied by the hospital pharmacy.

Effective doses of rhAngiostatin in mice were found in the range of 1.5–50 mg/kg/day (4.5–150 mg/m²/day), and no toxicity was observed in mice and cytomolgous monkeys for doses up to 150 and 300 mg/m²/day, respectively (EntreMed). With these data in mind, 7.5 mg/m² (40-fold below the highest studied safe dose in cytomolgous monkeys, based on BSA) was considered to be a proper starting dose. On the basis of a maximum injection volume of 2 ml/time point, the largest feasible dose that could be delivered in a bid schedule by s.c. injection was defined at 30 mg/m²/day.

Patients self-administered rhAngiostatin s.c. in the area of the lower abdomen or upper leg after being carefully instructed by research nurses. In a few cases, a relative of the patient performed the injections. Any volume exceeding 1 ml was divided into two separate syringes, resulting in a maximum of two injections twice daily. Dosing was done at a 12-h intervals (±1 h). The first dose for each patient was administered during hospitalization, with frequent monitoring of vital signs and acute side effects. After the first dose, patients were observed in the hospital for 24 h. At-home treatment compliance was monitored by interview during the outpatient visits, and by collecting and counting used syringes.

Analytic Procedures
Pharmacokinetic Sample Collection and Data Analysis. Levels of rhAngiostatin protein in human sera were determined by ELISA (EIA) after depletion of Plg by immunoadsorption. A murine MAb directed at the proteinase domain of human Plg, 3644, was obtained from American Diagnostica, Inc. (Greenwich, CT). The antibody was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) as recommended by the manufacturer. MAb 3644 was coupled with a yield of >94% and a substitution of 4.7–5.2 mg MAb/ml drained. For depletion of Plg, the insoluble MAb 3644 was used at a concentration of 2.5 mg/ml of suspension. Human serum samples were diluted 1:100 with PBS containing 0.1% Tween 20, and 0.5 ml of sample was incubated overnight at 4°C with 0.1 ml of insolubilized MAb 3644. The sample was microcentrifuged for 5 s, and the supernatant was removed for determination of rhAngiostatin. Immulon 4 plates (Dynex Technologies, Inc., Chantilly, VA) were coated overnight at 4°C with 0.05 ml antihuman Plg MAb (10 μg/ml; Calbiochem, San Diego, CA). After three washes with PBS and 30-min incubation at room temperature with 0.2 ml 3% nonfat dried milk, plates were washed three times and incubated for 60 min at room temperature with 0.05 ml of standard rhAngiostatin sample or PBS diluted serum samples containing 0.1% Tween 20 (PBS/Tween). The plates were then washed three times with PBS/Tween and incubated for an additional 30 min at room temperature with 0.05 ml of 1 μg/ml goat antihuman Plg IgG-PO ( Peroxidase; Cedarlane Laboratories, Ontario, Canada). The plates were then washed three times with PBS/Tween and developed using 0.05 ml ABTS peroxidase substrate (Kirkgaard & Perry Laboratory, Inc., Gaithersburg, MD). Data, performed in triplicate, were analyzed by the use of SOFTmax PRO software programs integral to the SpectralMAX 250 plate reader (Molecular Devices Corp., Sunnyvale, CA).

Concentration of rhAngiostatin was determined in serum samples of patients at day 1 before dosing and at 0.5, 1, 1.5, 2, 4, 6, 8, 12, 16, and 24 h after dosing (omitting the second dose at day 1), and at day 28 before dosing followed by 0.5, 1, 1.5, 2, 4, 6, 8, and 12 h after dosing. In 5 patients receiving 30 mg/m²/day, sampling was expanded to 10, 14, 16, 18, 20, 22, and 24 h after first dosing at day 28 (omitting the second dose of that day, too). Four patients were sampled after >8 months of treatment; sampling was done every 2 h up to 32 h after initial injection, whereas drug administration was temporally omitted. On days 15, 28, 35, every 15th day of each 4-week treatment cycle and at study termination a predose trough level was assessed.

Pharmacokinetic parameters [volume of distribution (Vd/F), clearance (Cl/F), serum half-life (T1/2), and AUC(0–infinity)] were assessed using noncompartmental analysis with WinNonlin 3.0 Professional (Pharsight Corporation, Mountain View, CA). Baseline levels of endogenous angiostatin in patient pretreatment serum were subtracted in all of the subsequent samples collected after rhAngiostatin administration. This was done to be able to estimate the pharmacokinetic values attributable to exogenously administered drug. For calculation of pharmacokinetic values at day 28 rhAngiostatin serum levels before dosing at day 28 were subtracted.

Antibody Formation. Antigenic properties of rhAngiostatin were measured in patient serum samples before starting treatment and every month thereafter. End point titers of anti-rhAngiostatin IgG and IgM antibodies in patient sera were determined by ELISA (EIA). Immulon 4 plates (Dynex Technologies, Inc.) were coated for 2 h at room temperature with 0.05 ml rhAngiostatin (2 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6; Sigma, St. Louis, MO). The coating solution was aspirated and blocked for 30 min at room temperature with 0.2 ml PBS containing 3% nonfat dry milk. The blocking medium was aspirated, and 0.05 ml patient serum was serially diluted in triplicate in PBS/Tween beginning with 1:200. After a 1 h incubation at room temperature, the wells were aspirated and washed three times with 0.2 ml PBS containing 0.1% Tween 20. The plates

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were tapped dry and incubated for 30 min at room temperature with 0.05 ml of either a 1:4000 dilution of goat anti-human IgG horseradish peroxidase (Kirkegaard & Perry Laboratory, Inc., Gaithersburg, MD) or goat antihuman IgM horseradish peroxidase (Kirkegaard & Perry Laboratory, Inc.). The wells were then washed three times with 0.2 ml PBS/Tween, and 0.05 ml ABTS peroxidase substrate (Kirkegaard & Perry Laboratory, Inc.) was added. After 30 min incubation, the absorbance at 405 nm was determined. The data were analyzed to determine the end point titer of immunoreactivity. End point titer was defined as the inverse dilution resulting in a 2-fold increase over baseline absorbance. If 1:200 dilution of serum resulted in an absorbance that was no greater than the negative controls, the titer of the serum was defined as 0.

**Angiogenic Factors.** Levels of the angiogenic growth factors VEGF and bFGF were determined in urine and plasma of all patients by ELISA (R&D Systems, Abingdon, Oxon, United Kingdom), following the manufacturer’s guidelines. Levels were measured in duplicate before treatment, at day 28 and 35, every first day of each 4-week treatment cycle, and at study termination. Obtained absorbance values were plotted against standard curves generated on the ELISA plate with correlation coefficient of >0.95. Relative changes of protein levels compared with pretreatment levels were calculated.

**RESULTS**

**Patient Characteristics.** All of the patients met the inclusion criteria, and were assessable for toxicity and pharmacokinetics at day 1. One patient discontinued treatment after 8 days; all of the other patients completed the first 4 treatment weeks. rhAngiostatin was administered for >4000 patient-days to 24 patients in three different doses. Male:female ratio was 10:14 and median age was 53 years. Table 1 summarizes the demographic data, ECOG performance at baseline, tumor types, number of previous systemic therapies, and treatment days. Seven patients had lung cancer, 5 patients had colorectal cancer, 3 patients had ovarian cancer, 2 had breast cancer, and 7 patients had other primary solid tumors.

**Toxicities.** rhAngiostatin was well tolerated at all of the dose levels. After prolonged treatment, exceeding 12 months in 5 patients, no new toxicities were noted. Table 2 lists grade 3 and 4 toxicities reported during the complete rhAngiostatin treatment period. Few grade 1 and 2 toxicities were reported during treatment. An exception is the occurrence of transient, localized, erythematosquamous lesions around the injection site (Fig. 1, A and B) without itching, pain or specific histology (Fig. 1C) occurring after 1–4 weeks of treatment. In affected patients ~50% of the injections resulted in skin lesions. Lesions diminished within 2–3 weeks during continued treatment and did not reappear. This toxicity was seen in 13 patients (in 11 patients grade 1 and 2 patients grade 2) in all three of the dose levels. Furthermore, 6 patients reported fatigue (3 grade 1, 3 grade 2) and 6 patients had complaints of nausea (5 grade 1, 1 grade 2).

One breast carcinoma patient developed DVT of the leg 8 days after starting rhAngiostatin treatment. This was attributed to high tumor load and prolonged immobility. She also suffered from dyspnea as a result of excessive pleural effusion. The patient was taken off study and not replaced. Another breast carcinoma patient with extensive skin metastases developed DVT of the arm during the second treatment month. A causal relation with rhAngiostatin could not be excluded, and treatment was discontinued.

Two patients suffered from bleeding in brain metastases. One patient with synovia sarcoma was diagnosed with bleeding metastases after 2 months of rhAngiostatin treatment but had documented episodes of bleeding in lung metastases also before start of rhAngiostatin treatment. The other patient (adenocarcinoma of the lung) was diagnosed with hemorrhage in a brain metastasis after 2 months of rhAngiostatin treatment just 2 weeks after radiotherapy for bone metastases. Both patients discontinued rhAngiostatin treatment. Another patient with adenocarcinoma of the lung developed symptomatic brain metastases, but in this case no hemorrhagic event was noted. At that time, 14 patients remained on trial. They were offered the option to undergo a CT scan to screen for brain metastases. Four patients chose to undergo a scan; no additional brain metastases were found. No relevant changes from baseline were observed in coagulation tests during

| Table 1 | Patient characteristics | n = 24 |
| --- | --- | --- | --- |
| Characteristic | Median | no. | % |
| Age, years | 53 | 34 |
| Range | 43-75 | |
| Sex | Male | 10 | 42 |
| Female | 14 | 58 |
| Primary tumor | Lung | 7 | 29 |
| Colon | 5 | 21 |
| Ovarium | 3 | 13 |
| Breast | 2 | 8 |
| Other | 7 | 29 |
| ECOG performance | 0 | 15 | 62 |
| 1 | 9 | 38 |
| Number of systemic therapies | Median | 2 | |
| Range | 0-8 | |
| Treatment days | Median | 98 | |
| Average | 176 | |

| Table 2 | Toxicities during rhAngiostatin treatment |
| --- | --- | --- |
| Event | Toxicity grade |
| Bleeding brain metastasis | 3 | 2 |
| DVT | 2 |
| Second primary cancer | 1 |
| Dyspnea | 2 |
| Hypertension | 2 |
| Diarrhea | 2 |
| Anemia | 1 |
| Lymphopenia | 1 |
| Hyponatremia | 1 |
rnAngiostatin treatment in all 24 of the patients (data not shown).

In 2 patients hypertension was noted while undergoing rhAngiostatin treatment. This was asymptomatic and could easily be controlled with mono-drug therapy.

The diarrhea in 1 patient was caused by gastroenteritis that had also occurred before initiating rhAngiostatin treatment. The other patient with diarrhea was later diagnosed with peritoneal metastases of his pancreatic carcinoma.

The anemia, lymphopenia, and hyponatremia occurring in 3 different patients were considered to be effects of cancer progression.

**Pharmacokinetic Parameters.** Baseline endogenous levels of angiostatin were measured in all 24 of the patients. Mean serum concentration (±SD) was 0.044 ± 0.014 μg/ml. Fig. 2 shows serum levels of rhAngiostatin after s.c. injection. No accumulation of rhAngiostatin occurs between days 1 and 28, (Fig. 2, A and B), and the shape of the time versus serum level curves is similar at both days. C max was reached after 2 h in all of the patients. There is no change in clearance during treatment; trough levels (taken directly before repeat drug administration) in subsequent treatment months are comparable with those found in the first month. Also after >8 months of treatment the serum levels remain predictable in 4 patients treated with 7.5 mg/m²/day (Fig. 2C). Trough rhAngiostatin serum levels for patients treated with 7.5 mg/m²/day were 0.32 ± 0.08 μg/ml; for 15 mg/m²/day 0.55 ± 0.23 μg/ml; and for 30 mg/m²/day 1.03 ± 0.32 μg/ml.

Within individual patients, trough levels followed in time had a SD ranging from 4 to 28%. Pharmacokinetic parameters are shown in Table 3. At the range of doses evaluated, the dose of rhAngiostatin shows, when administered s.c., a linear relation with AUC(0-8) \( r = 0.921 \) (day 1) by linear regression analysis for individual data, \( P < 0.0001 \), Fig. 3A, and \( r = 0.833, P < 0.0001 \) (day 28, data not shown)] and \( C_{\text{max}} \) \( r = 0.913 \) (day 1) by linear regression analysis for individual data, \( P < 0.0001 \), Fig. 3B, and \( r = 0.842, P < 0.0001 \) (day 28, data not shown]). By comparing \( \text{AUC}_{(0-8)} \) in patients receiving 15 mg/m²/day or 30 mg/m²/day s.c., with patients receiving these drug doses i.v. in a previous Phase I study.
PK Study of rhAngiostatin by s.c. Injection

Table 3 Pharmacokinetic parameters

<table>
<thead>
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<th>Dose level (mg/m²)</th>
<th>Study day</th>
<th># pts</th>
<th>C₀ (µg/mL)</th>
<th>Cmax (µg/mL)</th>
<th>T₁/2 (h)</th>
<th>Vd/F (L/m²)</th>
<th>CI/F (L/h/m²)</th>
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<td>7.5</td>
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<td>1.60 (±0.44)</td>
<td>3.17 (±0.54)</td>
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<td>0.42 (±0.26)</td>
<td>10.6 (±3.3)</td>
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<td>7.5</td>
<td>28</td>
<td>7</td>
<td>0.34 (±0.096)</td>
<td>2.47 (±0.35)</td>
<td>2.62 (±0.64)</td>
<td>1.10 (±0.12)</td>
<td>0.31 (±0.098)</td>
<td>13.2 (±4.2)</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>8</td>
<td>0.045 (±0.014)</td>
<td>2.80 (±0.60)</td>
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<tr>
<td>15</td>
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<td>8</td>
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<td>3.18 (±0.59)</td>
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<td>30</td>
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<td>8</td>
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<td>5.23 (±1.08)</td>
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<td>5.77 (±0.92)</td>
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<td>1.86 (±0.42)</td>
<td>0.54 (±0.10)</td>
<td>28.6 (±5.7)</td>
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(14), bioavailability (F) of rhAngiostatin after s.c. injection was estimated to be ~70%.

BSA of patients varied between 1.38 and 2.16 m² (median 1.91 m²). Clearance (Cl/F) was 0.69 ± 0.24 liter/h. A relation between BSA and CI/F (not normalized for BSA) was analyzed by calculating Pearson and Spearman correlations. Pearson correlation coefficient (r = 0.109, P = 0.46) and Spearman correlation coefficient (ρ = 0.206, P = 0.16) indicated no significant relation.

Antibody Formation. rhAngiostatin is a recombinant protein cloned from the human genome with a single altered amino acid (13). Patients were monitored for immune reactions against the agent. No clinical signs of systemic allergic reactions were seen during or after s.c. injection with rhAngiostatin, except for skin toxicity. However, biopsies of skin lesions in 2 patients did not show clear signs of immune-mediated events. Every month serum from patients was evaluated for antibody formation against rhAngiostatin. Twenty-two of 24 patients had, during the complete treatment period, IgG antibody titers at or below 1:200. In 1 patient with moderately large erythematous reactions at injection sites, serum analysis showed a slight rise in IgG antibody titer (1:1,600). Another patient developed large infiltrates on both legs (grade 2) without itching after ~1 month of treatment. She turned out to have high IgG antibody titers (1:25,600). Despite continued drug administration, the infiltrates spontaneously resolved completely, although the titer remained high. No IgM antibodies were detected in any of the three dose cohorts with serum titers at or below 1:200. We did not investigate interaction of rhAngiostatin with other proteins.

Angiogenic Factors. In ~90% of plasma and urine samples, bFGF and VEGF levels were detectable. rhAngiostatin treatment did not influence bFGF or VEGF levels in urine or plasma in our study population (data not shown). However, pretreatment values were higher in patients that progressed during treatment when compared with patients with stable disease.

Patient Follow-Up and Antitumor Effect. A median treatment of 99 days (range, 8–517 days) was completed in 24 patients. None of the patients had an objective tumor response according to WHO criteria. One patient had a minor response [30% reduction of tumor volume (abdominal metastases of gall bladder cancer) at 9 months]. Long-term stable disease (>6 months) according to standard WHO criteria (16) was observed in 6 of 24 patients. Unfortunately, the tumor growth rate of all the patients before starting rhAngiostatin treatment was unavailable.

One patient was retrospectively diagnosed with a distal colon polyp as seen in the CT evaluation before start of treatment. During rhAngiostatin treatment, the metastases of his testicular carcinoma remained stable, and there was a slight increase in the size of the colon mass, which, after biopsy, was diagnosed as adenocarcinoma. Patient refused surgery and was continued on treatment, but at evaluation after 15 months of treatment, liver metastases had appeared.

We were unable to evaluate 2 patients for tumor response: 1 because of radiation therapy on the whole pulmonary tumor, and the other because of study termination after 8 days. In the remaining 22 patients median time to progression according to
rhAngiostatin. had adverse effects from withdrawal or restart with a maximum delay of 14 days. None of the patients were observed from radiotherapy or after retreatment with rhAngiostatin treatment. For 3 of these patients radiotherapy was given for painful metastases. One patient received radiotherapy on her lower back for bone metastases, and 2 patients received rib and lower pelvis irradiation, respectively, enabling tumor measurement at sites distant from the radiation field. In the fourth patient the primary (marker lesion) tumor was irradiated to prevent bronchial obstruction, disabling additional assessment of tumor response. rhAngiostatin treatment was discontinued 1–3 days before and restarted 1–3 days after radiotherapy. No signs of unusual adverse effects were observed from radiotherapy or after retreatment with rhAngiostatin.

Five patients interrupted treatment for holiday reasons with a maximum delay of 14 days. None of the patients had adverse effects from withdrawal or restart with rhAngiostatin.

DISCUSSION

This study primarily demonstrates that rhAngiostatin, at doses between 7.5 and 30 mg/m²/day given in a bid schedule by s.c. injections, was well tolerated in a group of 24 patients for a total of >4000 treatment days. At the range of doses evaluated in this study, rhAngiostatin showed linear pharmacokinetic behavior when administered by s.c. injection. This remains to be the case with prolonged use. With the current formulation, the maximum clinically achievable s.c. dose could be given with good tolerance. Establishing a maximum tolerated dose was not the primary objective of this study. The predominant treatment-related side effect was erythema at the injection site. We observed two hemorrhages in brain metastases during rhAngiostatin treatment, leading to discontinuation of treatment in the affected individuals. One of the affected patients had a history of bleeding episodes in lung metastases before rhAngiostatin treatment. At this stage it remains possible that a causal relationship with rhAngiostatin treatment exists, although the reported overall incidence of bleeding in brain metastases in cancer patients is ~15% (17, 18). rhAngiostatin did not induce changes in an extensive panel of coagulation parameters in the study population. Clearly this observation should be additionally monitored in future studies. These events resulted in adjustment of the eligibility criteria. Before starting rhAngiostatin therapy, a CT scan of the brain is now mandatory. A similar reasoning holds for the observed thrombotic events in 2 patients with progressive cancer (19). Antiangiogenic agents have been associated with increased incidence of vascular events. Pulmonary bleeding was observed in a Phase II trial for non-small cell lung cancer, combining carboplatin/paclitaxel with a monoclonal VEGF antibody (20). Furthermore, a high incidence of thromboembolic events occurred in a Phase I trial combining SU5416, a tyrosine-kinase VEGF-receptor blocking agent with gemcitabin and cisplatin (21). Taken together, future studies with these agents, including rhAngiostatin, should include a careful monitoring of the coagulation system. Before treatment, it is advisable to screen patients for preexistent cardiovascular disease and, as already implemented in this study, for brain metastases.

The increased number of patients per cohort allowed reliable conclusions on the formation of antibodies against rhAngiostatin. Although it is not currently known if these antibodies neutralized the biological effect of rhAngiostatin (in vivo or in vitro), titers did not appear to influence the pharmacokinetic parameters in both patients. Trough rhAngiostatin serum levels in patients with detectable antibody formation remained comparable with their rhAngiostatin serum levels during the first treatment month and to levels in patients receiving the same drug dose, who did not produce antibodies. In this study doses were calculated for BSA. Although a limited amount of patients has been assessed, no relationship between BSA and CI/F was found in this study. This may imply that future dosing can be done without BSA adjustment. This finding should be additionally investigated in larger populations.

We were able to follow 5 patients for >1 year, and observed no signs of cumulative toxicity. We observed stable disease (<25% increase in tumor size) for >6 months in 6 of 24 patients, and in 1 patient a minor response.

Previous studies with antiangiogenic agents show that long-term therapy with an antiangiogenic agent may be necessary to observe significant antitumor effect (22). Even initial progression may occur before initiation of regression (23). A late response or beneficial effects of antiangiogenic treatment may be missed if regular rules for treatment discontinuation are applied. Antiangiogenic drugs such as rhAngiostatin will most likely be effective as an uninterrupted prolonged treatment (months to years) and not as an interval treatment. Thus, the protocol of this study used adjusted WHO criteria for progression of disease: a progression of 100% in tumor size compared with baseline or occurrence of new lesions of >1 cm in diameter, and only patients with good performance (ECOG 0–1) were included. We think that allowing an initial progression in tumor size ensures the registration of a delayed biological effect of a cytostatic agent and enables monitoring the long-term side effects of these drugs for potential chronic use. However, the
design of this study does not allow conclusions on a possible antitumor effect of rhAngiostatin.

In tumor-bearing mice, serum levels that showed antitumor effect were achieved with single daily s.c. injections of 1.5–50 mg rhAngiostatin/kg/day. Pharmacokinetic evaluation of this dose schedule in mice showed an AUC(0→∞) of 3.4 μg·h/ml (using 50 mg/kg/day, and is 150 mg/m²). Clearly, species differences are an important determinant of sensitivity to a drug. With this in mind, analysis of the drug levels in patients during the current study reveals a daily systemic exposure to rhAngiostatin in the same order of magnitude as observed in preclinical studies using biologically active doses.

The pharmacokinetic profile of this s.c. schedule is different from single daily i.v. bolus infusions (14). i.v. administration of rhAngiostatin showed linear pharmacokinetics with a serum half-life of 20 min. This implies that patients are exposed to rhAngiostatin for only a short period of time. And to achieve a continuous exposure to rhAngiostatin of >1 μg/ml by single daily i.v. injection, a dose of 120 mg/m² was needed (with an AUC(0→∞) of ~400 μg·h/ml; Ref. 14), whereas this same exposure was reached with s.c. injections of 30 mg/m² in a bid schedule (reaching an AUC(0→∞) of ~40 μg·h/ml), thus preventing exposure of the patients to very high peak serum levels. In addition, the schedule and s.c. injections allows patients to administer the medication in the privacy of their own homes. In the absence of toxicity and surrogate markers for biological activity, at the present time it is difficult to recommend a dose for Phase II evaluation. When a more concentrated formulation of rhAngiostatin becomes available, dose escalation using the bid s.c. regimen should be considered.

In conclusion, at the doses used in this study, s.c. treatment with rhAngiostatin is feasible and safe even after prolonged use, and does not result in excessive antibody formation. By twice-daily s.c. dosing an AUC and trough levels were reached in patients that showed effect in preclinical models. These findings suggest that the s.c. route of administration of rhAngiostatin is preferred above the i.v. bolus route and merits continued evaluation.

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