**Cancer Therapy: Clinical**

Phase I Clinical and Magnetic Resonance Imaging Study of the Vascular Agent NGR-hTNF in Patients with Advanced Cancers (European Organization for Research and Treatment of Cancer Study 16041)

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**Abstract**

**Purpose:** This phase I trial investigating the vascular targeting agent NGR-hTNF aimed to determine the (a) dose-limiting toxicities, (b) maximum tolerated dose (MTD), (c) pharmacokinetics and pharmacodynamics, (d) vascular response by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), and (e) preliminary clinical activity in solid tumors.

**Experimental Design:** NGR-hTNF was administered once every 3 weeks by a 20- to 60-minute i.v. infusion to cohorts of three to six patients with solid tumors in escalating doses. Pharmacokinetic and pharmacodynamic analyses in blood were done during the first four cycles. DCE-MRI was done in cycle 1 at baseline and 2 hours after the start of the infusion.

**Results:** Sixty-nine patients received a total of 201 cycles of NGR-hTNF (0.2-60 μg/m²). Rigors and fever were the most frequently observed toxicities. Four dose-limiting toxicities were observed (at doses of 1.3, 8.1, and 60 μg/m²), of which three were infusion related. The MTD was 45 μg/m². The mean apparent terminal half-life ranged from 0.963 to 2.08 hours. DCE-MRI results of tumors showed a vascular response to NGR-hTNF. No objective responses were observed, but 27 patients showed stable disease with a median duration of 12 weeks.

**Conclusions:** NGR-hTNF was well tolerated. The MTD was 45 μg/m² administered in 1 hour once every 3 weeks. DCE-MRI results showed the antivascular effect of NGR-hTNF. These findings call for further research for defining the optimal biological dose and clinical activity of NGR-hTNF as a single agent or in combination with cytotoxic drugs. 

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Translational Relevance

By the use of dynamic contrast-enhanced magnetic resonance imaging, this phase I study shows, for the first time in humans, that treatment with the vascular targeting agent NGR-hTNF causes an antivascular effect in tumors. It also defines the maximum tolerated dose of NGR-hTNF, 45 μg/m², and shows that NGR-hTNF is well tolerated. These findings call for further research for defining the optimal biological dose and clinical activity of NGR-hTNF as a single agent or in combination with cytotoxic drugs.

dose-related shedding of soluble TNFα receptors I and II, acting like physiologic attenuators, is observed. In this way, they not only protect the body from harmful effects of TNFα but also limit TNFα bioavailability and antitumor activity.

Efficacy of cytotoxic treatment is typically evaluated by measuring volumetric changes of tumors, according to the Response Evaluation Criteria in Solid Tumors criteria (10). However, agents targeting the (neo)vasculature may not cause rapid involution of tumors. This is especially relevant because the biological active dose may be considerably lower than the maximum tolerated dose (MTD). Therefore, NGR-hTNF efficacy should be assessed by also evaluating the effect of NGR-hTNF on tumor vasculature.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is a noninvasive imaging technique that can be used to measure properties of the tumor microvasculature in vivo. Imaging methods sensitive to the presence of the contrast agent in the extravascular extracellular space can provide information on microvessel perfusion, permeability, and extracellular leakage space (11). DCE-MRI has been introduced previously in phase I studies to assess the pharmacodynamic response to vascular targeting agents (12–18).

Here, we present the results from a phase I study, first in human, with i.v. administration of NGR-hTNF. The objectives of this study were (a) to determine the MTD, (b) to characterize the dose-limiting toxicities (DLT), (c) to investigate the pharmacokinetics and pharmacodynamics, (d) to measure the vascular response to NGR-hTNF by DCE-MRI, and (e) to determine preliminary clinical activity.

Patients and Methods

Patient selection. Eligibility criteria included patients ages ≥18 y with histologic or cytologic evidence of advanced cancer not amenable to established forms of effective therapy; Eastern Cooperative Oncology Group performance status ≤2; prior radiotherapy, chemotherapy, or hormonal therapy complete ≥4 wk before study enrollment; life expectancy ≥12 wk; and adequate bone marrow (platelets >100,000/μL, absolute neutrophil count

>1,500/μL), hepatic (total bilirubin ≤1.5 × upper limit of normal, and aspartate aminotransferase and alanine aminotransferase <2.5 × upper limit of normal or <5 × upper limit of normal in case of hepatic metastases), and renal (serum creatinine ≤1.5 × upper limit of normal) functions. All patients gave written informed consent. The study was approved by the medical ethical committee.

Drug administration and study design. This was an open-label, nonrandomized, multinational phase I dose escalation trial of NGR-hTNF conducted at two sites. NGR-hTNF was provided by MolMed. Before infusion, it was diluted to the appropriate concentration with 0.9% NaCl containing human serum albumin.

NGR-hTNF was administered once every 3 wk by a 20-min i.v. infusion during the four first dose levels and, at dose level 5, by a 1-h i.v. infusion to try to minimize chills during infusion. The starting dose was 0.2 μg/m². The number of dose levels was not fixed in advance. The trial was conducted in two stages: stage 1, accelerated phase and pre-DLT phase; stage 2, DLT stage. The dose escalation process was based on the severity of toxicity observed at each dose level. In stage 1 at the accelerated phase, only one patient per dose level was treated. After observation of grade 2 related toxicity, the pre-DLT phase started with three patients per dose level. As soon as a DLT was observed, the DLT stage started, in which cohorts of six patients were studied. Successive dose levels were defined as DLα = DL(n−1) × 2 (0.4-0.8-1.6... μg/m²) until mild related toxicity (grade <2) and afterward according to a modified Fibonacci. Without affecting the accelerated stage of the first dose levels, two additional patients were entered at the first four dose levels to perform DCE-MRI. In order not to compromise safety, patients 2 and 3 of these dose levels were included only when the patient at the higher dose level had completed cycle 1 of the treatment. After the accelerated phase, dynamic imaging remained optional but at least one to two patients every two dose levels were subject to dynamic imaging.

The incidence and severity of adverse events were scored according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (version 3). Response to therapy was monitored by Response Evaluation Criteria in Solid Tumors. NGR-hTNF–related DLT was assessed at cycle 1 and defined as follows: any grade 3 or 4 nonhematologic toxicity (except for nausea, vomiting, and fever that can be rapidly controlled with appropriate measures), grade 4 neutropenia for ≥7 d, febrile neutropenia, grade 4 thrombocytopenia or thrombocytopenic bleeding requiring transfusion, or severe hypotension requiring dopamine administration. These acute toxicities had to be declared related by the clinical investigator to be taken into account in the decision rule. The MTD was defined as the highest dose at which not more than one DLT was observed among a maximum of six patients. The cohort size at the MTD was expanded to 10 patients.

Pretreatment and follow-up studies. Complete history, physical examination, and routine laboratory studies, including complete blood count, electrolytes, renal and
hematologic assessments, and data analysis. Pretreatment measurements were done weekly. DCE-MRI data acquisition and analysis. The tissue relaxivity of Gd-DOTA was assumed to be homogeneously distributed.

Other imaging data were used to assess whether the number of pixels with values equal to zero, which may be regarded as fitting errors or as necrotic tumor parts. Back transformation of these log-transformed average values resulted in average values of $T_{\text{Vlow}}$ and $T_{\text{Vhigh}}$ from the formula $T_{\text{Vhigh}} = M + 1.96 \times SD$, where $M$ and SD are the mean and the SD values. Then, the numbers of pixels that were below and above $T_{\text{Vhigh}}$ were determined for the pharmacokinetic parameters, both at baseline and 2 h after the administration of NGR-hTNF.

Statistical analysis. Safety and efficacy data were summarized using appropriate descriptive statistics. A paired $t$ test was done to assess whether the number of pixels with values of $k_{\text{ep}}$ and $K_{\text{trans}}$ above and below the predetermined

### Table 1. Patient characteristics ($n = 69$)

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<td>Prior treatment, n (%)</td>
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<td>Tumor types, n (%)</td>
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<td>Colorectal cancer</td>
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<td>Kidney cancer</td>
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<tr>
<td>Lung cancer</td>
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<tr>
<td>Other</td>
<td>10 (14.5)</td>
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Abbreviation: ECOG, Eastern Cooperative Oncology Group.

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Phase I NGR-hTNF and DCE-MRI

Pharmacokinetic sampling, pharmacodynamic determinations, and data analysis. Pharmacokinetic and pharmacodynamic blood sampling was done on day 1 with samples drawn before infusion and at 20, 60, 90, 120, 180, 240, and 360 min after the start of infusion for NGR-hTNF and on day 21. NGR-hTNF antibodies and soluble plasma TNF receptors (sTNF-RI and sTNF-RII) were computed by using an ELISA. Maximum plasma concentration ($C_{\text{max}}$) and area under the plasma concentration time curve up to the last detectable concentration ($AUC_{\text{0-t last}}$) were estimated from plasma concentration time data using standard noncompartmental methods.

DCE-MRI data acquisition and analysis. DCE-MRI was done in patients with either primary or metastatic tumors in the liver or the head and neck region. DCE-MRI data were acquired at baseline, within 1 wk before start of NGR-hTNF, and 2 h after start of the first administration of NGR-hTNF. This 2-h interval was chosen because a maximal synergism between NGR-hTNF and cytotoxic agents has been observed with a 2-h delay between NGR-hTNF and drug administrations, suggesting a maximum effect of NGR-hTNF on the vasculature at that time point (19).

Measurements were done on a 1.5-T magnetic resonance system (Siemens), using a body phased array coil. After conventional anatomic imaging, 15 mL of 0.5 mol/L Gd-DOTA (Dotarem) were administered i.v. with an injection rate of 2.5 mL/s by a Spectris magnetic resonance injection system. Using a T1-weighted fast low-angle shot (FLASH) sequence with a time resolution of 2 s, Gd-DOTA uptake in the tumor was monitored and the bolus passage in vessels in the spleen or the carotid artery was monitored. Sequence parameters were repetition time, 50 ms; echo time, 4.4 ms; flip angle, 45°; and slice thickness, 7 mm, four to six slices. DCE-MRI data were acquired for 90 s.

Just before Gd-DOTA injection, proton density–weighted images were recorded with the same sequence parameters as the DCE-MRI except for a flip angle of 8° and a repetition time of 200 ms. Data from these images were combined with the DCE-MRI data to calculate the concentration of Gd-DOTA, using the method described by Hittmair et al. (20). The tissue relaxivity of Gd-DOTA was assumed to be homogeneously distributed.

For the analysis of the DCE-MRI data, our previously described method was used (21, 22), with automated detection of a vascular normalization function from pixels in the spleen in case of liver tumors and from pixels in the carotid artery in case of head and neck tumors. The spatial distribution of the values of the rate constant $k_{\text{ep}}$ and the volume transfer constant $K_{\text{trans}}$ were represented in a map. On a T1-weighted magnetic resonance image, recorded directly before Gd-DOTA arrival in the tumor, regions of interest were drawn, which comprised all tumor tissue in the field of view. These regions of interest were applied to the maps of $k_{\text{ep}}$ and $K_{\text{trans}}$ to select the single values of $k_{\text{ep}}$ and $K_{\text{trans}}$ for all tumor pixels. The means of $k_{\text{ep}}$ and $K_{\text{trans}}$ of the pixels of all slices containing tumor tissue were calculated after log transformation. Log transformation excluded all pixels with values equal to zero, which may be regarded as fitting errors or as necrotic tumor parts. Back transformation of these log-transformed average values resulted in average values of $k_{\text{ep}}$ and $K_{\text{trans}}$ for the whole tumor (21). In addition, histograms of the log-transformed pixel values of $k_{\text{ep}}$ and $K_{\text{trans}}$ were analyzed (23). For all tumor tissue of each patient, a low threshold value ($TV_{\text{low}}$) was calculated at baseline from the formula $[TV = M - 1.96 \times SD]$ and a high TV from the formula $[TV_{\text{high}} = M + 1.96 \times SD]$, where $M$ and SD are the mean and the SD values. Then, the numbers of pixels that were below $TV_{\text{low}}$ and above $TV_{\text{high}}$ were determined for the pharmacokinetic parameters, both at baseline and 2 h after the administration of NGR-hTNF.
threshold significantly changed after treatment compared with baseline.

Results

General. Seventy patients were included in the study. One patient, on dose level 16, never started treatment due to a gastrointestinal bleeding grade 3 after registration and before start of treatment. This patient was replaced. All baseline characteristics, protocol treatment, and safety sections are described on the safety population \((n=69)\). Patient characteristics are listed in Table 1. They received a total of 201 cycles of NGR-hTNF at doses ranging from 0.2 to 60 \(\mu\)g/m\(^2\). The median number of cycles administered was two (range, 1-12).

In Table 2, the dose escalation schedule and the DLTs are described. In total, four DLTs were observed, of which three were infusion related. The MTD for NGR-hTNF was 45 \(\mu\)g/m\(^2\) in 1 hour, once per 3 weeks.

Adverse events. In Table 3, all hematologic, biochemical, and nonhematologic adverse events during cycle 1 and over all cycles are summarized. Rigors and fever during infusion were the most frequently observed toxicities. Despite prolonging the duration of the infusion from 20 minutes to 1 hour from dose level 5 (1.95 \(\mu\)g/m\(^2\)) onward, grade 1 and grade 2 chills did not diminish. Overall, the frequency and the grade of toxicities did not differ between the various dose levels and only mild toxicity was observed.

Pharmacokinetic and pharmacodynamic analyses. Pharmacokinetic analysis was done in all patients. The mean apparent terminal half-life ranged from 0.963 to 2.08 hours. The apparent volume of distribution \((V_s)\) of NGR-hTNF encompasses total body water, suggesting extensive distribution and/or binding of NGR-hTNF to tissue. Following a 1-hour infusion of NGR-hTNF over the dose range of 0.8 to 60 \(\mu\)g/m\(^2\) NGR-hTNF, the exponent of the power model was 1.30 (95% confidence interval, 1.24-1.36) for \(C_{\text{max}}\), and 1.26 (95% confidence interval, 1.20-1.31) for \(AUC_{0-4}\), indicating a slightly greater than dose-proportional relationship (Fig. 1A and B).

At low dose (<1.3 \(\mu\)g/m\(^2\)), the levels of sTNF-RI and sTNF-RII were scattered around zero and thereafter increased proportionally with dose \((r^2 = 0.53, P = 0.0014\) and \(r^2 = 0.61, P = 0.0003\), respectively), with an apparent plateau observed at \(\geq 25\) \(\mu\)g/m\(^2\) \((Fig. 2)\). Anti-NGR-hTNF antibodies were not detected.

Antitumor activity. No objective responses were observed. However, 27 patients (39%) showed stable disease with a median duration of 12 weeks and a range between 5 and 35 weeks. Six patients received at least six cycles: one patient on 0.2 \(\mu\)g/m\(^2\) (6 cycles), one patient on 0.8 \(\mu\)g/m\(^2\) (6 cycles), two patients on 1.3 \(\mu\)g/m\(^2\) (6 and 7 cycles), one...
patient on 1.95 μg/m² (12 cycles), and one on 14.36 μg/m² (6 cycles).

DCE-MRI. Thirty-six patients were scheduled to undergo a DCE-MRI. Three did not undergo MRI due to logistical reasons, and in two patients, the acquired data were incomplete. Therefore, in 31 patients, DCE-MRI data were available for analysis. Twenty-six patients had tumors/metastases localized in the liver and five patients had tumors in the head and neck region. The administered dose of NGR-hTNF ranged from 0.2 to 45.0 μg/m² according to the predefined protocol.

In 12 patients, a change in $k_{ep}$ in tumor tissue was observed after NGR-hTNF administration, which was larger than the repeatability coefficient that was determined in a previous study (Fig. 3A; ref. 21). The repeatability coefficient is a statistical measure, providing direct insight into the probability that differences between two measurements are due to real differences in, for example, tumor biology and not due to the measurement protocol (24). In selected patients, a decrease in the rate constant $k_{ep}$ in liver metastases after NGR-hTNF could even be observed by visual inspection of the color-coded maps of the spatial distribution of the values of $k_{ep}$ (Fig. 3B and C). At doses from 1.3 to 25.4 μg/m², the mean $k_{ep}$ and $K^{trans}$ significantly decreased ($P < 0.01$), whereas at lower and higher dose levels, no statistically significant changes in mean $k_{ep}$ and $K^{trans}$ were observed. On average, in the histogram analysis, the percentage of pixels of $k_{ep}$ and $K^{trans}$ below the lower threshold value significantly increased in all dose levels 2 hours after administration of NGR-hTNF ($P < 0.01$; Fig. 3D). The percentage of pixels of $k_{ep}$ and $K^{trans}$ above the higher threshold value did not change significantly ($P > 0.1$).

There was no relation between pretreatment values of $k_{ep}$ and $K^{trans}$ and the number of cycles of NGR-hTNF that the patients received. In addition, the change in $k_{ep}$ and $K^{trans}$ 2 hours after the administration of NGR-hTNF was not related to the number of cycles of NGR-hTNF that were administered.

Discussion

This is the first report on the systemic treatment of humans with NGR-hTNF. NGR-hTNF is well tolerated, as reflected by the relatively mild side effects even at high dose levels. The most frequently observed toxicities are short-lived rigors, which even occurred despite a more than doubling of the infusion time. Three of four DLTs were infusion-related reactions. Although, theoretically, these chills and infusion-related reactions could have been caused by the NGR peptide, it is more likely that cytokines were induced by the TNFα part of the drug. Preclinical data have shown that the NGR motif is poorly immunogenic, even
Fig. 3. A, the absolute change in $k_{ep}$ at baseline and 2 h after the start of administration of NGR-hTNF for individual patients at each dose level is plotted. Dashed line, repeatability coefficient (see text for details). B, spatial distribution of the values of the rate constant $k_{ep}$ in liver metastases and normal liver tissue in one slice before treatment with NGR-hTNF. C, spatial distribution of the values of the rate constant $k_{ep}$ in liver metastases and normal liver tissue in one slice 2 h after treatment with NGR-hTNF; arrows, tumor areas with decreased values of $k_{ep}$. D, results of the histogram analysis, indicating that the percentage of pixels of $k_{ep}$ below the lower threshold value before and 2 h after the start of administration of NGR-hTNF significantly increased (*, $P < 0.01$). The percentage of pixels of $k_{ep}$ above the higher threshold value did not significantly change ($P > 0.1$).
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<td>Gr 2</td>
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<tr>
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<tr>
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**NOTE:** a to d are four DLTs as described in Table 2.

**Abbreviations:** AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase.
when it was conjugated to TNF-α or to highly immunogenic carrier proteins (25). At dose level 17, 60 μg/m² in 1 hour, two DLTs were observed during the infusion of NGR-TNF. We did not prolong the infusion time any further as we hypothesized that—assuming a dose-proportional (linear) increase of Cmax and AUC of NGR-hTNF and a plateau of sTNF receptors at higher dose levels—the excess of free drug (unbound to circulating receptors) at higher doses would be responsible for systemic toxicities (DLTs). This hypothesis was based on the previous findings using very high doses of TNF in an isolated limb perfusion approach (26). Therefore, further prolongation of the infusion time was not expected to alter toxicity.

The MTD of NGR-hTNF administered in 1 hour was 45 μg/m² once per 3 weeks. It is difficult to compare our results with those obtained with untargeted recombinant TNFα because different administration schedules and infusion times have been applied. Nevertheless, in 19 phase I trials with recombinant TNF, the MTD ranged from 150 to >800 μg/m² (27–45), which is at least thrice higher than the MTD we found in our study. However, in mice, murine NGR-TNF was 12 to 15 times more effective than murine TNFα with similar toxicity (7).

The effect of NGR-hTNF on tumor vasculature was assessed by DCE-MRI. In the overall population, the histogram analysis of DCE-MRI data confirmed the antivascular activity of NGR-hTNF. Although results from the histogram analysis, showing activity in all dose levels, are most sensitive to minor changes in the vasculature induced by NGR-TNF, changes in mean values, suggesting a more consistent effect at some dose levels, may also be relevant as a larger number of pixels (i.e., tumor tissue) is involved. Therefore, further DCE-MRI studies on larger and more homogeneous patient populations are highly recommended for dose selection. Ideally, DCE-MRI could be used for dose finding, thus preventing unnecessary exposure of patients to higher dose levels. However, it should be noted that up to now, no biomarker in humans has been proved to adequately reflect the antitumor activity of vascular targeting agents (46). As is common in a phase I study with a heavily pretreated patient population and a variety of tumor types, no objective responses were observed in our study. Given the fact that direct antivascular effects of NGR-TNF 2 hours after administration were observed by DCE-MRI in this population, it will be useful to incorporate DCE-MRI in a subsequent phase II trial to evaluate its exact role in response prediction.

A confounding variable in the current study is that the magnetic resonance protocol allowed for the assessment of tumor vascularity only at a restricted part of the body (i.e., part of the liver or the head and neck region). Therefore, intrapatient variability of tumor response at other metastatic sites could not be assessed. In addition, DCE-MRI was done only once, at 2 hours after the first dose. This informed us about the direct antivascular effect of NGR-hTNF, but the effect over time of repeated cycles could not be assessed. The lack of correlation between the direct antivascular effect of NGR-hTNF (measured by DCE-MRI) and disease control (measured by the number of administered cycles) suggests that a direct antivascular effect at first cycle is insufficient to predict subsequent treatment outcome. Thus, although the use of DCE-MRI is crucial to obtain a better insight into the biological activity of NGR-hTNF, this method cannot replace the conventional assessment of the MTD. Moreover, the observed nonlinear effect of different doses of NGR-hTNF on tumor vascularity as measured by DCE-MRI underscores the importance of studying the full dose range up to MTD to elucidate the working mechanism of NGR-hTNF (46).

In conclusion, NGR-hTNF was well tolerated. Its main toxicities are mild-to-moderate rigors and fever. The MTD is 45 μg/m² when administered in 1 hour once per 3 weeks. DLTs are mainly infusion related. Although DCE-MRI and pharmacodynamics could not be used to define an exact optimal dose, the antivascular effect observed by DCE-MRI and the increase in levels of sTNF receptors also suggest that doses lower than the MTD are promising and deserve to be explored in further studies with NGR-hTNF, either as a single agent or in combination with cytotoxic drugs. Phase I to II clinical trials are currently ongoing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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