Phase I and Pharmacokinetic Studies of CYT-6091, a Novel PEGylated Colloidal Gold-rhTNF Nanomedicine

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Abstract

Purpose: A novel nanomedicine, CYT-6091, constructed by simultaneously binding recombinant human tumor necrosis factor alpha (rhTNF) and thiolyated polyethylene glycol to the surface of 27-nm colloidal gold particles, was tested in a phase I dose escalation clinical trial in advanced stage cancer patients.

Experimental Design: CYT-6091, whose dosing was based on the amount of rhTNF in the nanomedicine, was injected intravenously, and 1 cycle of treatment consisted of 2 treatments administered 14 days apart.

Results: Doses from 50 μg/m^2 to 600 μg/m^2 were well tolerated, and no maximum tolerated dose (MTD) was reached, as the highest dose exceeded the target dosage of 1-mg rhTNF per treatment, exceeding the previous MTD for native rhTNF by 3-fold. The first 2 patients on the study, each receiving 50 μg/m^2, did not receive any prophylactic antipyretics or H2 blockade. A predicted, yet controllable fever occurred in these patients, so all subsequently treated patients received prophylactic antipyretics and H2 blockers. However, even at the highest dose rhTNF's dose-limiting toxic effect of hypotension was not seen. Using electron microscopy to visualize nanoparticles of gold in patient biopsies of tumor and healthy tissue showed that patient biopsies taken 24 hours after treatment had nanoparticles of gold in tumor tissue.

Conclusions: These data indicate that rhTNF formulated as CYT-6091 may be administered systemically at doses of rhTNF that were previously shown to be toxic and that CYT-6091 may target to tumors. Future clinical studies will focus on combining CYT-6091 with approved chemotherapies for the systemic treatment of nonresectable cancers. *Clin Cancer Res; 16(24); 6139–49. ©2010 AACR.*

Tumor necrosis factor alpha (TNF) became available in recombinant form in the mid-1980s and remarkable antitumor effects were observed in experimental animals. However, numerous human clinical trials revealed that humans are exceedingly sensitive to the toxic side effects of recombinant human TNF alpha (rhTNF) and contemporaneous research in the 1980s identified this protein as a primary mediator of endotoxic shock and sepsis (1–5). The main dose-limiting toxicities (DLT) of intravenous recombinant rhTNF were hypotension, hepatotoxicity, and malaise and fatigue (1–5). The results of multiple phase II studies using intermittent bolus intravenous injections of rhTNF were reported between 1989 and 1992 (6–10). In more than 156 evaluable patients, there was only 1 complete and 1 partial response at the maximum tolerated dose (MTD). These disappointing results may, in part, be due to the inability to achieve therapeutic doses at the site of disease.

Limiting biodistribution of rhTNF primarily to tumors by hyperthermic limb perfusion for in-transit melanoma or sarcoma was shown to be dramatically successful when used in combination with interferon and melphalan (11, 12). When administered via isolation perfusion to the extremity, the overall response rates with rhTNF followed by melphalan ranged from 75% to 100% (1, 13–16). The responses were observed against a range of histologies, including melanoma, carcinoma, and sarcoma, and highlight the fact that when rhTNF is administered in a manner that limits its biodistribution primarily to tumor and is administered prior to chemotherapy, the antitumor effects

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Translational Relevance

The following article presents the first-in-man clinical trial of a unique nanomedicine, CYT-6091, which is comprised of recombinant human tumor necrosis factor alpha (rhTNF) bound to the surface of PEGylated 27-nm colloidal gold particles. The significance of this clinical study is multifold. First, we achieved doses of rhTNF formulated as CYT-6091 that exceed previous clinical trials with rhTNF by 3-fold and are equal to that given for the isolated limb perfusion (ILP) for in-transit melanoma or sarcoma, where 85% complete remission rates are routinely observed. Second, the tumor targeting seen in this study suggests that mimicking the ILP protocol with CYT-6091 administered systemically prior to chemotherapy might result in similar response rates. And last, as an intact tumor neovasculature is critical for this nanomedicine to target tumors, then the treatment of solid tumors should begin with nanomedicine administration prior to surgery, even for resectable tumors.

are reminiscent of those reported in preclinical animal models (17).

This observation serves to underscore the critical importance of balancing efficacy against toxicity and the role of targeting therapy to the site of tumor growth. The maximum, safe-tolerated rhTNF doses administered in isolation perfusion of the limb is 4 mg, which is 10-fold higher than that tolerated by intravenous administration (11–16). However, lowering the perfused rhTNF dose from 4 mg to 1 mg resulted in local response rates that are equivalent to that seen with 4 mg, indicating the benefit of limiting rhTNF's biodistribution to the tumor site (18, 19).

But, rhTNF alone has minimal antitumor effects in regional limb perfusion. Rather, it is the combination of rhTNF followed by chemotherapy where dramatic antitumor effects have been observed. The temporal sequence of treatment in this protocol is critical, as rhTNF has been shown to target tumor-associated vasculature, inducing hyperpermeability of the tumor blood vessels, leading to extravasation of erythrocytes and massive hemorrhagic necrosis of the tumor (20, 21). However, this hemorrhagic necrosis alone has not been shown to result in significant and sustained tumor reduction. Rather, it has been proposed that the rhTNF induction of hyperpermeability in the tumor is what enhances the efficacy of subsequently administered chemotherapy, resulting in such significant response rates with regional perfusion.

For tumors that are not able to be surgically isolated, improved response rates to chemotherapy with prior treatment with rhTNF might be accomplished by nanoparticle delivery systems capable of escaping phagocytic clearance by the reticuloendothelial system (RES; refs. 22, 23). By avoiding the RES, nanotherapeutics can circulate in the blood for longer periods of time. Under these conditions,

such delivery systems permit preferential extravasation into the inherently leaky tumor-associated vasculature and facilitate drug accumulation within the tumor (24–26). By design, nanoparticles capable of sequestering a cancer drug within a tumor may also reduce the accumulation of the drug in healthy organs, increasing the relative efficacy or safety of a cancer therapy and increasing its therapeutic index.

In this study, we used colloidal gold as the core of a delivery system for tumor-targeted drug delivery (27). Colloidal gold is a nanoparticle that is ideally suited for the delivery of protein biologics, such as rhTNF, because it binds these molecules very avidly and preferentially through available thiol groups (27). The long history of clinical use of colloidal gold in the treatment of rheumatoid arthritis and the prior use of radio-gold colloids in various human therapeutics, indicate that colloidal gold particles may be a safe platform for a new drug delivery system (28–31).

Preclinical studies with CYT-6091, a colloidal gold, rhTNF and thiolayted polyethylene glycol (PEG) construct, indicate that PEGylation of the gold nanoparticles avoids immediate uptake by the RES, reduces the toxicity of rhTNF, and allows the nanomedicine to sequester in solid tumors. This sequestration in tumors, which is thought to be due to the leakiness of the tumor neovasculature, increased over time, whereas, in contrast over this same time period, rhTNF levels declined in blood and other vital organs. In TNF-sensitive tumor models, CYT-6091 had a dramatic effect on reducing tumor burden, but in TNF-insensitive tumor models CYT-6091 was much less effective. In those models, combination treatment with CYT-6091 followed by chemotherapy was effective (32).

Here, we present the first clinical trial utilizing a nanoparticle to target rhTNF directly to the tumor, via systemic delivery. In this single agent trial in patients with advanced cancer, CYT-6091 was well tolerated and an MTD was not reached. Biological effects of rhTNF were noted as evidenced by predictable pyrogenic and hematologic changes. Pretreatment with antipyretics was used to manage fever, whereas the hematologic effects resolved themselves within 24 hours after treatment. In addition, core biopsies, taken 24 hours after treatment, revealed the presence of gold nanoparticles in tumor tissue.

Patients and Methods

Patient selection

Between May 2006 and April 2008, 30 patients with advanced and/or metastatic, histologically documented solid organ cancers, no longer considered responsive to available conventional modalities or treatments were enrolled in this phase I study. Patients were 18 years of age or older, with a clinical performance status of 0 to 1 on the Eastern Cooperative Oncology Group (ECOG) scale and life expectancy of greater than 3 months. Eligible patients had adequate hematologic function (platelet count $\geq 100 \times 10^9$/L, absolute neutrophil count

$\geq 1.5 \times 10^9$/L, and hemoglobin ≥ 10 g/dL), adequate renal and hepatic function [serum creatinine ≤ 2.0 mg/dL, alanine transaminase and aspartate transaminase ≤ 1.5-fold the upper limit of normal (≤ 10-fold the upper limit in presence of liver metastases), bilirubin ≤ 2.5 mg/dL] and adequate pulmonary function. Subjects with a history of pulmonary disease must have adequate pulmonary reserve, as evidenced by FEV1 greater than 30% predicted. Eligible patients were also required to have had no treatment with cytotoxic agents or treatment with biological agents within 3 weeks prior to treatment or within 6 weeks of prior treatment with nitrosureas and were to have fully recovered from toxicities of any prior treatment with cytotoxic drugs, radiotherapy, surgery, or other anticancer modalities. All patients signed an informed consent approved by the Institutional Review Board of the National Cancer Institute.

Treatment

This was a phase I, dose-escalation trial of CYT-6091. The following dose levels were evaluated: 50 μg/m^2 ($n = 3$), 100 μg/m^2 ($n = 3$), 150 μg/m^2 ($n = 3$), 200 μg/m^2 ($n = 3$), 250 μg/m^2 ($n = 3$), 300 μg/m^2 ($n = 4$), 400 μg/m^2 ($n = 3$), 500 μg/m^2 ($n = 3$), and 600 μg/m^2 ($n = 4$). Two doses (day 1 and 15) constituted 1 course. CYT-6091 was reconstituted in sterile water for injection by the National Institutes of Health Pharmacy Department personnel and delivered to the patient care unit (PCU) or intensive care unit (ICU) in a syringe labeled with the dose, volume, and time of reconstitution. It was then administered through a central catheter as a single IV push injection for no more than 20 to 30 seconds followed by 15 to 20 mL of 0.9% sodium chloride injection flush. IV hydration was initiated prior to treatment and maintained for 4 hours postinjection at a rate of 100 mL/h.

The patients were monitored for 48 hours after treatment. The first patient of each cohort received his/her first dose of CYT-6091 in the ICU. If acute toxicities were not observed, additional patients could receive the drug administration on the intermediate care unit (or ICU as determined by staffing requirements), with telemetry and pulse oximetry during the first 4 hours after dosing. Patients remained hospitalized for at least 48 hours, but could be discharged at that time if clinically stable. During and following each dose, vital signs (temperature, pulse, blood pressure, and respiration rate) were obtained at 5 minutes and then every 15 minutes for 60 minutes, then at 1.5, 2, 2.5, 3, 4, and 8 hours, then routinely if stable. After discharge from the hospital, patients were followed as outpatients. After 2 weeks (± 2 days), provided the patients had not experienced DLT and all toxicities had reversed to a grade 2 or less, they received a second dose of CYT-6091 on the PCU. A month following the second dose of CYT-6091, patients returned to clinic for restaging studies and final evaluation of toxicity. No intrapatient dose escalation or dose reduction was allowed.

If a patient had stable disease or a partial response at the time of evaluation (4 weeks after completing 1 course of therapy), then the patient was eligible for up to 3 retreat-

ment courses with the same dose and schedule that had been given safely before.

DLTs were defined as: 1) all grade 4 toxicities with the exception of grade 4 myelosuppression of 5 days or less duration unless accompanied by a fever greater than or equal to 38.5°C for longer than 24 hours; 2) all grade 3 toxicities with the exception of: i) grade 3 transaminase elevations occurring within the first week and resolving by day 15, ii) grade 3 constitutional symptoms that persist for less than 72 hours, iii) grade 3 myelosuppression of less than 5 days duration, or iv) grade 3 metabolic/laboratory events that are correctable within 24 hours; or 3) hypotension associated with oliguria or the requirement for pressors to maintain adequate urine output or blood pressure. The MTD was defined as the highest dose that induced DLT in no more than 1 patient among a cohort of 6 patients. If a DLT occurred in 1 of 3 patients accrued to a cohort, then that cohort would have been expanded to 6 patients. If another DLT occurred, then that cohort would have exceeded the MTD.

Assessments

Adverse events were graded according to the NCI-Common Terminology Criteria for Adverse Events (CTCAE version 3.0). Treatment-related toxicities were those toxicities considered by the investigators to be possibly, probably, or definitely related to CYT-6091. Response was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST; ref. 33).

Pharmacokinetics

The pharmacokinetic (PK) profile of CYT-6091 was determined using heparinized whole blood samples collected at pretreatment and at 5, 15, 30 minutes, and 1, 2, 3, 4, 8, and 24 hours following the completion of the first infusion of CYT-6091. PK analysis was performed for total rhTNF as it is not possible to assay for bound versus free rhTNF, nor is it possible to quantify the amount of gold based on the sensitivity of available assay techniques. The blood samples were serially diluted in assay diluent and analyzed using a sandwich ELISA (CytImmune Sciences, Inc.) specific for rhTNF. The potency estimates were further analyzed using a PK program (WinNonLin; Pharsight Corporation) to estimate C_{max}, Volume of distribution (V_d), area under the curve (AUC), and terminal half-life. The data are reported as the mean $\pm$ SD for each cohort.

Tissue biopsies and electron microscopy

Biopsies for the assay of colloidal gold particles were obtained using either palpation and transcutaneous core biopsy or CT (computed tomography)-guided core biopsy. The maximal effective radiation dose of 2 tissue biopsies, using CT scanning was 0.234 rem. This exposure was within the Radiation Safety Committee Guidelines for Adults (i.e., 5.0 rem). Other biopsies could be obtained during the second cycle of therapy. Where possible, the tumor was to be completely excised, along with a margin of normal skin or adjacent tissue. Only cutaneous lesions or

superficial lymph nodes, which could be removed with local anesthesia (with or without sedation), were completely excised. Lesions identified for possible excision during the course of treatment were labeled as nontarget lesions. In the case of core biopsies (i.e., liver), a biopsy of both tumor and normal tissue was obtained. Electron microscopy (EM) biopsy tissues were fixed in standard gluteraldehyde fixative and stored at 2ºC to 8ºC. High-power photomicrographs were obtained to examine for the presence of colloidal gold nanoparticles after the tissue had been embedded, sectioned, and stained using standard osmium tetroxide staining techniques. When possible, specimens from both malignant and normal tissue were obtained contemporaneously.

Immunogenicity

The immunogenicity of CTY-6091 was assessed via an assay for anti-rhTNF immunoglobulin G (IgG) titers from blood samples obtained at protocol-specified time points. For this immunologic assay, a 10 mL red-top vacutainer tube was used to collect a blood sample prior to the first and second dose of CYT-6091, and at week 6, the time of tumor restaging evaluation. The samples were stored on ice (or in a 4ºC refrigerator) and processed within 1 hour after collection, as follows. Blood samples were centrifuged for 10 minutes at room temperature and $2,400 \times g$. The resultant supernatant serum was separated and stored at -80ºC for assay of anti-rhTNF antibodies (IgG). A direct enzyme-linked immunosorbent assay (ELISA) was used to determine the IgG titers to rhTNF. For this assay, rhTNF antigen, human IgG or human serum albumin was coated onto the wells of an ELISA plate and incubated overnight. The following day, the plates were blocked with 1% PEG for 2 hours and washed again. Subject samples, collected at pretreatment, 2, and 6 weeks posttreatment, were diluted 1:5, 1:50, and 1:250 in assay diluent (2% Albumin, 0.1% Tween diluted in Tris-buffered saline) and added to designated wells of rhTNF-coated ELISA plates with mouse anti-rhTNF or rabbit-anti-rhTNF (R&D Systems, Inc. or CytImmune Sciences, Inc, respectively) diluted in assay diluent being used as positive controls. The plates were then incubated overnight. The following day the plates were washed and alkaline phosphatase-conjugated, species-specific goat antibodies, diluted in assay diluent, were added to the appropriate wells. The plates were incubated for an additional hour, washed once again followed by the addition of substrate. The plates were read at a wavelength of 405 nm once the optical density (OD) for the mouse positive control reached 1.4 (at which point the rabbit positive control had an OD of about 2.5). A positive antibody response was defined as a 20-fold difference in signal between the pretreatment sample and the posttreatment samples for each patient at a 1:250 dilution.

Statistical analysis

To evaluate whether there was any consistent effect on blood pressure, changes in systolic and diastolic blood pressure (BP) measurements were evaluated in treated subjects by time-point following each infusion, evaluating first changes in the population of treated subjects as a whole and then in dose cohorts divided into thirds as follows: low dose (50, 100, and 150 μg/m^2), mid dose (200, 250, and 300 μg/m^2), and high dose (400, 500, and 600 μg/m^2). Changes for each time-point were compared with baseline by a t test.

Results

Patient characteristics

Thirty patients were enrolled in the protocol and 29 were treated. One patient with an ampulla of Vater adenocarcinoma was enrolled; however, this patient suffered a stroke prior to receiving any treatment. Patients with a variety of histologies were treated; however, most had ocular melanoma ($n = 9$) or adenocarcinoma of the colon ($n = 8$). The rest of the cohort was composed of 3 patients with adenocarcinoma of the pancreas, 2 patients with ductal carcinoma of the breast, and 1 patient of each of the following histologies: melanoma, adenocarcinoma of the lung, adenocarcinoma of the rectum, leiomyosarcoma, adrenocortical carcinoma, soft tissue sarcoma, and 1 of an unknown primary histology. Most patients had at least 1 prior systemic regimen before enrolling on this study. The median time on treatment was 43 days. Most patients received 2 doses of CYT-6091 (1 course); 1 patient received 2 courses and 1 patient received 4 courses. Two patients received a single cycle of CYT-6091 and were taken off study prior to the second dose due to disease progression. Of the 29 patients treated, 26 were taken off study due to disease progression, 2 patients refused further treatment, and 1 patient died of his disease.

Adverse events and response assessment

The treatment was well tolerated and there were no DLTs. The first 2 patients treated on the study did not receive any prophylactic antipyretics or H2 blockade, to accurately evaluate the toxicity profile. However, a predicted, yet controllable fever occurred in these patients and all patients subsequently treated received prophylactic antipyretics and H2 blockers. Table 1 lists all grade 3 and 4 adverse events reported by dose level, regardless of attribution. The most frequent grade 3 and 4 adverse events were lymphopenia (26/29 patients), hypoalbuminemia (5/29), hypokalemia (5/29), hypophosphatemia (5/29), hyperbilirubinemia (5/29), and increased AST (aminotransferase; 5/29). In addition, a predictable (34) redistribution in circulating lymphocytes and neutrophils that was dose dependent was seen (Fig. 1). All events resolved per protocol requirements and were not considered DLTs. The main DLTs of intravenous recombinant rhTNF in previous trials were hypotension and hepatoxicity. Administration of doses up to 600 μg/m^2 of CYT-6091, exceeding our target dosage of 1-mg rhTNF per treatment, did not result in hypotension (Fig. 2) or in hepatoxicity. All clinical responses, including blood pressure and hematologic measures, at all doses following the

Table 1. Grade 3 and 4 adverse events experienced by dose level

Adverse event (CTCAE v 3.0)	50 µg/m^2 (n = 3)	100 µg/m^2 (n = 3)	150 µg/m^2 (n = 3)	200 µg/m^2 (n = 3)	250 µg/m^2 (n = 3)	300 µg/m^2 (n = 4)	400 µg/m^2 (n = 3)	500 µg/m^2 (n = 3)	600 µg/m^2 (n = 4)	Total (n = 29)
Blood/bone marrow										
Lymphopenia	2	3	1	3	3	4	3	3	4	26
Leukocytes (total WBC)				1					1	2
Hemoglobin							1		1	2
Neutrophils/granulocytes									1	1
Metabolic/laboratory										
Hypoalbuminemia			1		1	1	1		1	5
Hypokalemia			1					2	2	5
Hypercalcemia				1					1	2
AST/SGOT				1	1	1			2	5
ALT/SGPT									1	1
Alkaline phosphatase				1						1
Hypophosphatemia					2	2	1			5
Hyperbilirubinemia								1	4	5
Adverse events										
Fatigue	1	1				1				3
Pain, chest wall	1									1
Pain, liver		1								1
Pain, abdomen			2	1			1			4
Pain, bone				1						1
Pain, head									1	1
Pain, NOS							1			1
Nausea						1	1			2
Vomiting						1	1			2
Diarrhea							1			1
Infection, pneumonia							1			1
Infection, urinary					1		1			2
Anorexia						1				1
Thrombosis/embolism								1		1

NOTE: AE numbers represent the highest AE grade per person for all injections received, regardless of attribution.
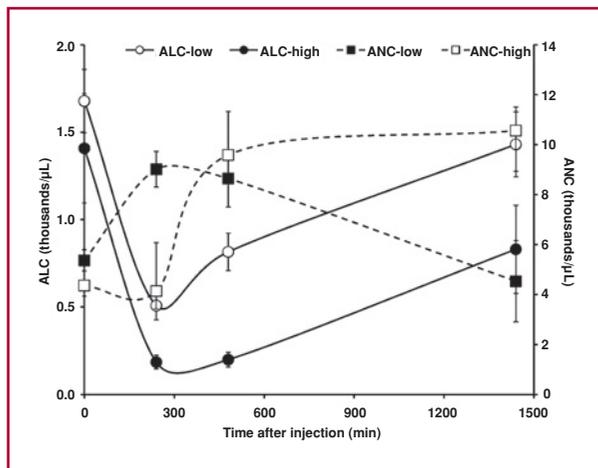
Fig. 1. CYT-6091 induces a predictable redistribution of lymphocytes and neutrophils. All patients from all the dosing groups of CYT-6091 were sorted into 3 dose cohorts: High (400, 500, 600 $\mu g/m^2$), Medium (200, 250, 300 $\mu g/m^2$), and Low (50, 100, 150 $\mu g/m^2$). Data are presented as the mean ± SEM for the counts from 2 of the 3 dosing cohorts. ALC, absolute lymphocyte count; ANC, absolute neutrophil count.

second dose of CYT-6091 were indistinguishable from those following the first treatment.

Of the 29 patients treated 62% (18/29) experienced at least 1 episode of hypotension following treatment (Fig. 2). There were statistically significant decreases in the mean systolic BP after both treatment infusions that initially occurred sporadically but then became consistent about 1 to 2 hours postdosing ($P < 0.001$). Analysis by dose cohort (low dose, medium dose, and high dose) suggested that the changes were not strongly dose-related in that although the high dose cohort showed the greatest mean decrease at 3, 4, and 8 hours ($P < 0.0001$, $P = 0.0009$, and $P < 0.0001$, respectively), this was not a consistent observation across all time points.
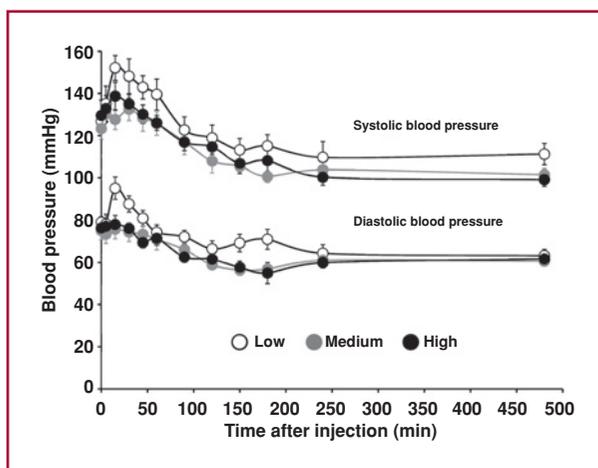

Fig. 2. Effect of CYT-6091 on blood pressure (BP). Patients were grouped into 3 cohorts as described in Figure 1. Data are presented as the mean ± S.E.M. Mean systolic BP did not drop below 80 mmHg, a threshold for hypotension requiring medical intervention.

This analysis also revealed, however, that even at the times when the largest decreases occurred, the resulting mean systolic BP values were still within the normal range (90 to <130 mmHg) and there were no subjects who received pressors in response to hypotensive episodes. Indeed, using the FDA toxicity grading scale for normal volunteers (FDA, 2007) only 2 subjects achieved a grade 3 hypotension (systolic BP <80 mmHg) and in both cases, these were single excursions, not requiring medical intervention.

Of the 27 of 29 subjects evaluated for disease response, 1 patient had a partial response by RECIST criteria, whereas 4 patients had stable disease. This former patient, who had melanoma and was treated with 100 $\mu g/m^2$, continued to respond through 3 cycles (7 months in duration). The 4 subjects, 1 each in the 100, 250, 300, and 600 $\mu g/m^2$ cohorts, respectively, all achieved stable disease in the first cycle. However, of these 4 subjects only the 1 in the 250 $\mu g/m^2$ cohort underwent a second cycle, at the end of which disease progression was observed. All of the remaining 22 evaluated subjects, that is, 76% of those receiving treatment, experienced disease progression.

Pharmacokinetics

Blood samples were taken at 5, 15, 30, 60, 120, 180, 240, 480, and 1,440 minutes for PK analysis and evaluated for rhTNF concentrations using the rhTNF ELISA detailed above. As indicated in Table 2, the concentration–time profiles showed gradually decreasing concentrations of rhTNF through 4 hours (480 minutes) after the start of the infusion for all cohorts and through 24 hours (1,440 minutes) for the 3 highest dose cohorts, that is, doses below 400 $\mu g/m^2$ produced no detectable levels of rhTNF at the last time point, that is, 24 hours postdosing. These data indicate that the plasma concentrations at all time points show a monotonic relationship with the dose administered.

As seen in Table 2, the half-life ($t_{1/2}$) data across all cohorts show no apparent relationship to dose and are all of the same order of magnitude, indicating that the removal of CYT-6091 follows a first-order reaction. AUC and Cmax values, however, increase with increasing dose in an approximately monotonic relationship to dose.

Electron microscopy of biopsied samples

Detection of the gold particles in tissue biopsy samples via transmission electron microscopy (TEM) was used as initial proof of concept of the tumor targeting ability of CYT-6091 (Table 3). Biopsies (punch, core, or excisional) were obtained from 20 subjects 24 hours post-administration of CYT-6091. Tissue samples were obtained from both tumor and adjacent healthy tissue. The samples were fixed in formaldehyde/glutaraldehyde, 2.5% each in 0.1 mol/L of sodium cacodylate buffer, pH = 7.4. Postfixation tissues were analyzed by TEM using standard osmium tetroxide staining techniques. Figure 3 presents a side-by-side comparison of normal and neoplastic breast tissue taken from a subject with invasive ductal carcinoma of the breast 24

Table 2. Mean ± SEM whole blood concentrations of rhTNF (ng/mL) following infusion with various dose levels of CYT-6091

Time, min	Dose administered, µg/m²								
	50	100	150	200	250	300	400	500	600
5	7.7 ± 0.1	17.2 ± 2.6	25.2 ± 1.3	47.6 ± 5.8	58.2 ± 16.4	62.6 ± 12.1	85.1 ± 11.9	106.7 ± 9.7	154.7 ± 34.7
15	5.2 ± 0.7	10.1 ± 1.5	16.5 ± 1.0	34.2 ± 5.6	43.8 ± 9.7	48.1 ± 13.6	74.9 ± 13.4	73.8 ± 15.8	147.5 ± 22.8
30	4.0 ± 0.6	6.8 ± 1.0	11.4 ± 1.9	25.9 ± 6.9	24.4 ± 4.8	33.3 ± 9.8	58.4 ± 12.2	60.0 ± 16.2	109.2 ± 16.1
60	2.5 ± 0.4	3.5 ± 0.3	6.5 ± 1.2	15.5 ± 4.7	21.7 ± 2.8	21.3 ± 6.7	46.5 ± 4.6	43.3 ± 13.3	90.2 ± 12.7
120	1.3 ± 0.3	1.7 ± 0.1	3.2 ± 0.7	8.4 ± 2.9	10.9 ± 1.5	10.9 ± 4.0	21.7 ± 4.5	24.6 ± 8.3	47.2 ± 3.4
180	0.9 ± 0.2	1.1 ± 0.1	1.8 ± 0.4	4.6 ± 1.3	4.7 ± 0.6	4.3 ± 1.4	11.8 ± 3.8	12.2 ± 3.8	24.0 ± 1.8
240	0.6 ± 0.1	0.7 ± 0.1	1.3 ± 0.3	3.1 ± 0.9	2.6 ± 0.4	2.4 ± 0.8	6.1 ± 1.6	8.9 ± 3.0	12.3 ± 1.1
480	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.2	1.0 ± 0.2	0.8 ± 0.0	0.8 ± 0.4	1.6 ± 0.5	2.6 ± 0.6	2.6 ± 0.0
1,440	BDL	BDL	BDL	BDL	BDL	BDL	0.3 ± 0.1	1.2 ± 0.1	0.6 ± 0.0

Analysis (mean ± SD) of the pharmacokinetic parameters of CYT-6091 in man

Dose of CYT-6091, µg/m²	Total dose, µg	C_{max}, ng/mL	Terminal half-life, min	AUC, min × ng/mL	Volume of distribution, mL	Clearance, mL/min
50	90 ± 5	7.7 ± 0.2	120.7 ± 19.6	611 ± 192	18,686 ± 2,988	155 ± 40
100	202 ± 33	17.2 ± 4.6	131.3 ± 21.0	977 ± 64	24,064 ± 8,308	209 ± 48
150	270 ± 49	25.2 ± 2.3	127.0 ± 34.4	1,575 ± 412	18,641 ± 2,845	177 ± 67
200	314 ± 7	47.7 ± 9.9	146.3 ± 22.2	3,789 ± 1,465	12,063 ± 5,174	90 ± 28
250	410 ± 62	58.2 ± 16.4	112.7 ± 27.2	3,888 ± 890	11,355 ± 3,536	107 ± 23
300	563 ± 45	62.6 ± 21.0	113.0 ± 46.2	4,403 ± 2,223	13,241 ± 4,637	159 ± 96
400	785 ± 148	85.1 ± 20.7	266.0 ± 79.0	9,297 ± 3,845	16,265 ± 9,021	97 ± 51
500	823 ± 153	106.7 ± 16.7	371.1 ± 133	11,206 ± 4,359	31,248 ± 27,137	84 ± 53
600	1,208 ± 214	154.7 ± 34.7	160.5 ± 52.0	17,730 ± 2,928	10,865 ± 5,666	71 ± 24

Abbreviation: BDL, below the limit of detection.

Table 3. Detection of gold nanoparticles in normal and neoplastic tissues from subjects treated with various doses of CYT-6091

Dose, $\mu g/m^2$	Patient ID	Diagnosis	Site of biopsy	Normal tissue Particles	Tumor tissue Fields	Particles	Fields
50	0601-01	Cutaneous melanoma	Chest Wall	10	10	91	6
	0601-02	Colon adenocarcinoma	Liver	2	6	13	11
	0601-03	Ocular melanoma	Liver	0	4	5	11
100	0601-04	Colon adenocarcinoma	Liver	0	5	13	10
	0601-05	Colon adenocarcinoma	ND	–	–	–	–
	0601-06	Ocular melanoma	Scalp	1	7	0	7
150	0601-07	Lung adenocarcinoma	Liver	0	6	33	5
	0601-08	Pancreatic adenocarcinoma	Liver	0	6	135	12
	0601-09	Pancreatic adenocarcinoma	Liver	2	5	0	5
200	0601-10	Invasive ductal carcinoma	Breast	0	5	167	5
	0601-11	Leiomyosarcoma	Muscle	3	4	31	10
	0601-12	Ocular melanoma	Liver	7	6	8	10
250	0601-13	Ocular melanoma	Muscle	19	7	0	4
	0601-15	Pancreatic adenocarcinoma	Liver	1	5	4	7
	0601-16	Ocular melanoma	Liver	0	5	0	5
300	0601-17	Colon adenocarcinoma	ND	–	–	–	–
	0601-18	Ocular melanoma	Liver	8	10	16	12
	0601-19	Ocular melanoma	Liver	15	6	5	5
	0601-20	Ocular melanoma	Liver	23	6	0	6
400	0601-21	Desmoplastic small round cell	Omentum	0	5	0	6
	0601-22	Rectal adenocarcinoma	Liver	27	7	10	5
	0601-23	Colorectal adenocarcinoma	Liver	78	6	0	5
500	0601-24	Ocular melanoma	Skin	0	8	1	9
	0601-25	Invasive ductal carcinoma	Liver	148	9	0	7
	0601-26	Colorectal adenocarcinoma	ND	–	–	–	–
600	0601-27	Desmoplastic small round cell	ND	–	–	–	–
	0601-28	Colorectal adenocarcinoma	ND	–	–	–	–
	0601-29	Colorectal adenocarcinoma	ND	–	–	–	–
	0601-30	Adenocarcinoma	ND	–	–	–	–

Abbreviation: ND, not determined.

hours following the first infusion of CYT-6091. The black particles seen in the tumor tissue are gold particles, indicating that the nanoparticles trafficked to the tumor, but not to healthy breast tissue. In other patients where healthy and cancerous liver tissue was taken, gold particles were also detected in healthy liver tissue, an anticipated clearance site. However, the data shown in Figure 3 are the clearest example of differential tissue distribution. Given the limitations of the analysis and our inability to fully quantify the number of gold particles as a function of tissue mass, targeting at this point may only be inferred.
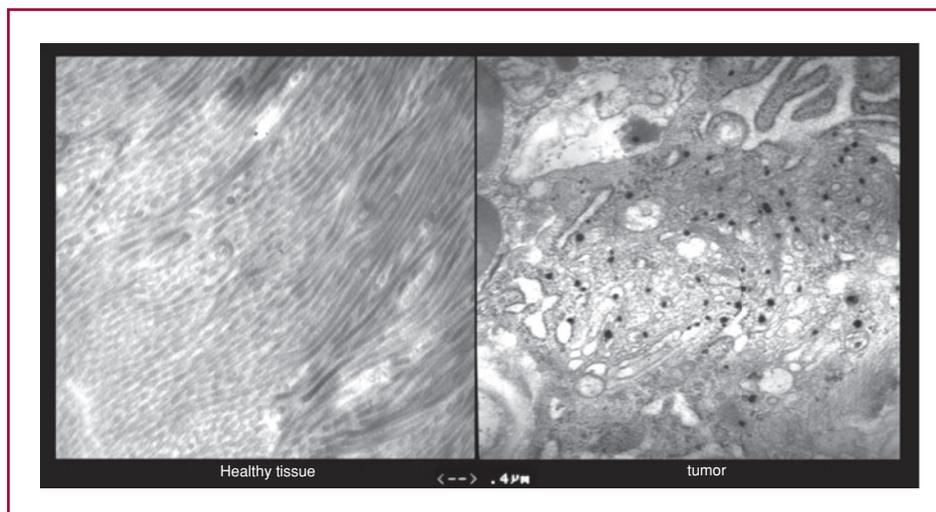
Discussion

Striking a favorable balance between efficacy and toxicity is a significant challenge for the development of new cancer therapies. The ideal agent is one that can be administered systemically yet exert its effects exclusively within the local tumor tissue. A variety of strategies have been attempted to achieve this goal. Nanomedicine, utilizing tumor-targeted particles carrying an antitumor agent, is one such strategy. In this study, we have evaluated the safety, PK, immunogenicity, and tumor-targeting capability of a novel nanomedicine, CYT-6091. CYT-6091 is a covalently linked PEGylated colloidal gold nanoparticle carrying rhTNF. On the basis of our preclinical studies, we found that CYT-6091 selectively trafficked to tumor tissue, delivered a therapeutic dose of rhTNF, caused massive vascular leak, and was safer and more effective than rhTNF (27, 35). Our results in this phase I clinical trial mirrored much of our preclinical findings.

Our dose escalation scheme was based on the dose of rhTNF as determined by the amount of rhTNF measured in the CYT-6091 final drug product. We were able to administer CYT-6091 in doses up to 600 $\mu g/m^2$ of rhTNF without encountering DLTs or immunogenicity. This dose exceeds the MTD of systemically administered, native rhTNF to patients and is within the range of

Fig. 3. Electron micrographs from a patient with inoperable ductal carcinoma of the breast. Magnification is 20,000×, and the line at the bottom is 0.4 µm. Black dots in the tumor micrograph are nanoparticles of gold.

effective rhTNF doses predicted by preclinical studies (1–4). In addition, we were able to demonstrate, by EM, accumulation of CYT-6091 in tumor tissue. Finally, although efficacy was not the primary endpoint of this study, we saw a partial response by RECIST criteria for a patient with metastatic ocular melanoma and stable disease with 4 others.

The PK analysis indicated that the half-life of rhTNF was approximately 5-fold longer for CYT-6091 than previous clinical trials with native rhTNF (∼130 minutes vs. ∼28 minutes, respectively). The rhTNF half-life observed in the present clinical study is similar to that seen in a rat push-pull cannula model where both rhTNF and gold were measured (36). In this model, the half-life for rhTNF and gold were quite similar (182 and 217 minutes, respectively), suggesting that the CYT-6091 nanoconstruct remains intact in the circulation. Further analysis of the PK data revealed that the AUC for the highest dose of CYT-6091 resulted in an exposure to rhTNF that is 4-fold higher than that seen with highest native rhTNF, but without any DLT (2–4).

Previous studies have suggested that the DLT of systemically administered rhTNF is primarily hypotension resulting from a direct effect of rhTNF on the endothelial cells that comprise normal, healthy blood vessels (37, 38). By having rhTNF bound to the colloid gold nanoparticles, we hypothesize that the biodistribution of rhTNF is restricted, improving the safety of higher doses of rhTNF, and most likely eliminating this toxic effect on endothelial cells. Further, the immunogenicity data indicate that no anti-TNF antibodies were detected 2 or 6 weeks after treatment, suggesting that the improved safety was not due to the neutralization of the CYT-6091 rhTNF by TNF-absorbing antibodies. And, of note is that systemically administered CYT-6091 induced fever and a retrafficking of lymphocytes, but did not induce hypotension, indicating that the CYT-6091 construct is able to separate rhTNF's clinically manageable responses from its DLT response.

Electron micrographs of the patient biopsies, taken 24 hours after treatment, indicate that the gold nanoparticles traffic to tumors. Such trafficking to the tumor is anticipated as the neovasculature of tumors has been shown to have fenestrations of 200 to 400 nm in size (20–24). At 27 nm, we hypothesize that CYT-6091 would be able to readily exit the circulation through the well-described enhanced permeability and retention (EPR) effect in tumor neovasculature. In addition, our preclinical studies indicate that rhTNF serves as an ideal tumor-targeting ligand, as gold particles with rhTNF on the surface were seen in the tumor within 30 minutes after treatment, whereas, in contrast, the same amount of gold nanoparticles without rhTNF bound to the surface took approximately 24 hours to be detected in the tumor (27). In addition, the targeting of CYT-6091 to tumors in preclinical mouse tumor xenograft models is independent of whether the tumor cells do or do not have TNF receptors (Paciotti, unpublished observations). Consequently, CYT-6091 may target solid tumors by both a passive mechanism (the EPR effect), as well as an active one (rhTNF binding to the tumor neovasculature).

It should be noted that many of these patients also had biopsies of normal liver, and gold nanoparticles were also seen in healthy liver tissue (Table 3). In contrast, when other tissues were biopsied, such as healthy skin or breast tissue, no gold particles were detected. These data are consistent with other preclinical studies, showing that 1 day posttreatment most of the gold administered as CYT-6091 was found in the liver, decreasing by 50% 120 days after treatment (39–41). It is possible that the PEGylation of the gold nanoparticles may keep the particles monodispersed, allowing for better elimination from the liver, which would suggest improved long-term safety of CYT-6091 compared with that seen with chrysotherapy.

Analysis of the EM data indicates that the number of nanoparticles accumulating in tumor tissue was independent of dose. Even at the lowest dose, nanoparticles of gold were detected in tumor biopsies, and at the higher doses of

CYT-6091, no proportional increase in nanoparticle accumulation was observed. Of note, 2 patients, one with ductal carcinoma of the breast and the other with pancreatic cancer, who did not have resections of their primary tumors, appeared to have the greatest number of particles in the biopsies of these primary tumors. This observation suggests that an intact tumor neovascualture may allow for better tumor targeting of these gold nanoparticles, further supporting the strategy of treating patients with nanomedicines prior to surgery.

The clearest clinical benefit of rhTNF is in hyperthermic limb perfusion for in-transit melanoma or sarcoma (11–19). In this protocol, rhTNF is infused first, followed 30 to 60 minutes later with the infusion of chemotherapy. Now as an EMA-approved treatment protocol, improvements have been developed since this procedure was described in 1992. Most significantly, the perfusion dose of rhTNF has been lowered from 4 mg to 1 mg, with no change in the percentage of patients responding, suggesting that a threshold may be reached where additional drug is of no further benefit (18, 19). The success of this surgical protocol, where a 70% to 90% complete response rate has been reported, is the ideal protocol design to mimic, but with systemic treatments using CYT-6091. Assuming CYT-6091 traffics preferentially to solid tumors, *a priori* the goal of this phase I study was to achieve systemic dosing of CYT-6091 of at least 1 mg of rhTNF per dose. At 600 $\mu g/m^2$, this objective was met. Now the efficacy of systemically admi-

nistered CYT-6091 needs to be tested in combination with chemotherapy.

A phase II clinical trial in patients with solid tumors that administers CYT-6091 systemically first, followed 30 to 60 minutes later with chemotherapy, replicates the isolated limb perfusion protocol. Future studies are planned to evaluate whether CYT-6091 induces greater vascular leak as determined by dynamic contrast-enhanced MRI and to evaluate the safety and efficacy of CYT-6091 in combination with approved chemotherapies to determine whether or not such a combination is not only safe, but improves the response rates of these chemotherapies.

Disclosure of Potential Conflicts of Interest

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