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Biodistribution of TNF- α -coated gold nanoparticles in an *in vivo* model system

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Abstract

Aim—In this study, we describe the biodistribution of CYT-6091, a colloidal gold (Au)-based nanomedicine that targets the delivery of TNF- α to solid tumors.

Materials & methods—A single intravenous injection of CYT-6091 coated with 5 μ g TNF- α was given to human prostate tumor-bearing or naive (without tumor) nude mice. Tissues were harvested and analyzed at specific time points for Au nanoparticles by atomic emission spectroscopy and TNF- α by ELISA.

Results—The two constituents of CYT-6091, TNF- α and Au, exhibited different behavior in blood, with TNF- α showing a faster decay than the Au nanoparticles. Between 0 and 4 h after injection, TNF- α showed a preferential accumulation in the tumor. Au was observed to accumulate preferentially in the liver between 4 and 12 h, and showed some clearance over time (4 months).

Conclusion—These data suggest that CYT-6091 delivers TNF- α preferentially to the tumor and that upon TNF- α degradation, the liver takes up Au, which is cleared slowly over time.

Keywords

biodistribution; cryosurgery; CYT-6091; gold nanoparticle; TNF

Nanomedicines currently being developed for the treatment of cancer, although diverse in chemical composition, size and shape, must address similar key physiologic and engineering barriers to warrant their testing in a clinical setting. First, these nanosystems must be engineered to avoid initial clearance and uptake by the reticuloendothelial system [1–3]. Second, the putative nanomedicines must alter the biodistribution of their therapeutic payload(s) to allow

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

preferential uptake at the site of disease (e.g., the solid tumor) while simultaneously avoiding uptake in healthy tissues. Finally, these nano-drugs must be manufactured to set specifications and in compliance with regulatory guidelines for current Good Manufacturing Practices.

CYT-6091 is a first-generation nanomedicine that is currently being evaluated in a Phase I clinical trial at the National Cancer Institute (NCI, USA). This nanotherapeutic is manufactured on a pegylated colloidal gold (Au) nanoparticle platform and is designed to target the delivery of human TNF- α to solid tumors [4]. The nanomedicine sequesters within solid tumors by passively extravasating from the leaky tumor vasculature and by actively binding to TNF- α receptors on the cells present in the tumor inter-stitium. In preclinical models, CYT-6091 has been shown to improve the therapeutic index of TNF- α treatment by increasing the safety and efficacy of TNF- α treatment [4,5].

We recently reported that CYT-6091 when used in combination with cryosurgery or hyperthermia resulted in a synergistic antitumor response when compared with either treatment alone [6–8]. Specifically, with cryosurgery, it was observed that the addition of TNF- α , administered as CYT-6091, increased the minimum temperature required for complete tumor destruction from -40 to -0.5°C . Similar experiments conducted with soluble TNF- α , while resulting in comparable responses, also lead to a 30% mortality rate [6]. Although short-term preclinical assessments of TNF- α confirm that the improved safety and efficacy of CYT-6091 treatment is due to the altered biodistribution of TNF- α , little is known about the short- and long-term distribution and clearance of the nanoparticles.

In the present study, we investigated the biodistribution of both TNF- α and Au particles following a single intravenous injection of CYT-6091 in tumor-burdened, athymic nude mice. The presence and/or clearance of Au particles over a longer time were also investigated for up to 120 days in naive (nontumor bearing) animals. Briefly, these data show a 90% loss in TNF- α activity in the blood within 4 h after injection, despite the presence of 60% of the injected nanoparticles. During this interval, TNF- α was observed to accumulate preferentially in the tumor. After 4 h, Au particles were found to accumulate preferentially in the liver (and equally in spleen on capacity per gram of tissue basis) up to a period of 24 h after injection, with minimal uptake in other healthy organs. The longer-term analysis over a 4-month period showed significant Au clearance from the liver.

Materials & methods

Cell culture

LNCaP Pro 5 cells were grown as adherent monolayers in 25 cm² T-flasks. The growth medium consisted of Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum and 10^{-9} M dihydrotestosterone.

Tumor cell implantation

LNCaP Pro 5 cells were trypsinized and resuspended in approximately 100 μl of MatrigelTM matrix (Matrigel diluted 3:1 in serum-free media; BD Biosciences, Bedford, MA, USA). A total of 2–3 million cells were subcutaneously injected in the hindlimb of athymic nude mice weighing 20 g as described previously [6]. Experiments were performed after 5–7 weeks when a tumor diameter of 7–8 mm was obtained.

Nanoparticle systems

Biodistribution studies were performed in two groups of nanoparticles. The first group consisted of the nanodrug, CYT-6091, which contained both poly(ethylene glycol) (PEG)-Thiol and TNF- α covalently bonded onto Au nanoparticles [4]. The second group comprised

the control nanoparticle, Au-PEG-Thiol (CYT-6091 without TNF- α), to investigate the role of TNF- α in the biodistribution.

Nanoparticle treatment

CYT-6091 was calibrated with respect to the relative amounts of TNF- α and Au. Each 1 μ g of Au is coated with approximately 40 ng of TNF- α (or 400 TNF- α molecules on each Au nanoparticle; data not shown) [4]. A single intravenous injection of CYT-6091 suspended in saline containing 5 μ g (~250 μ g/kg) of TNF- α or 125 μ g of Au (~6.25 mg/kg) was administered to tumor-bearing or naive animals as described later. Similarly intravenous injections of Au-PEG containing 125 μ g of Au were conducted in the control group. Animals were euthanized at selected time points, and blood and other tissues (e.g., tumor, spleen, liver, kidney and lung) were harvested and weighed.

Groups

The groups of animals used in the study are described in TABLE 1 and listed below:

- The biodistribution of CYT-6091, as assessed by its components (Au and TNF- α analyzed separately), was measured at several time points in animals with LNCaP Pro 5 tumors. Au analysis was performed by atomic emission spectroscopy (AES) at 5 min, and 1, 4, 12, 24 and 72 h, and TNF- α was assessed by ELISA at 5 min, and 1 and 4 h after injection of CYT-6091. Separate groups of animals were used for Au and TNF- α analysis to ensure adequate weight of each tissue. Three to four animals were used at each time point for both Au and TNF- α groups. Transmission-electron microscopy (TEM) was performed for detection of Au in the tumor, liver and spleen harvested 3 days after CYT-6091 injection.
- The biodistribution of Au was assessed by AES in animals injected with Au-PEG particles at 5 min, and 1, 4 and 24 h postinjection.
- Longer-term (up to 4 months) biodistribution and clearance studies of the Au component in CYT-6091 were conducted in naive animals (nontumor bearing) as the tumor burden precludes investigation beyond a few weeks in tumor-bearing mice. AES was performed at 1 h, and 1, 60, 90 and 120 days after CYT-6091 injection.

Animals were randomized before experiments and each group was composed of three to four animals.

AES for Au quantification

Gold quantification was performed using a Perkin-Elmer Optima 3000 DV atomic emission spectrometer. Approximately 0.2–0.3 g of each tissue (liver, kidney, tumor, lung, spleen and blood) was digested with 5 ml aqua regia (four parts concentrated HNO₃ + one part concentrated HCl). Before analysis, each sample was diluted 1:1 with a solution of 2 ppm yttrium in dilute nitric acid as an internal standard. Calibration of the instrument was performed using a solution of 1 ppm Au and 1 ppm yttrium in 50% aqua regia. Aliquots of this solution were analyzed by comparing them with a calibration curve constructed using multielement standards. Organ Au concentrations were normalized to total tissue weight.

ELISA for TNF- α quantification

At selected time points blood and other tissues were collected and flash frozen. To determine intraorgan human TNF- α content, the organs were later homogenized using a polytron tissue disrupter. Debris was removed by allowing the homogenate to stand on ice for 20 min. The supernatant was analyzed for TNF- α concentration for total protein using a commercial protein

assay (BioRad, Hercules, CA, USA) specific for human TNF- α . Organ TNF- α concentrations were normalized to total protein content in the tissues [4,7].

Electron microscopy

Electron microscopy was performed to verify the uptake of electron-dense Au nanoparticles by the tissues. Animals were sacrificed 3 days after the CYT-6091 injection and 1 mm³ liver, spleen and tumor samples were cut randomly and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 h. They were postfixed in 2% osmium tetroxide in the buffer for 2 h, washed and dehydrated in graded concentrations of ethanol and propylene oxide. Tissue samples were then embedded in Epon (Polysciences Inc, PA, USA) and thin-sectioned. Thin sections of 60–70 nm were collected on formvar copper grids and stained with 1:1 mix of methanol and lead citrate. The grids were visualized under JEOL 100 transmission-electron microscope (Hitachi, Japan).

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by multiple comparison Bonferroni's test. Data were collected from at least three different animals and $p < 0.05$ was considered statistically significant.

Results

Pharmacokinetic & biodistribution of CYT-6091 in tumor-bearing animals

The *in vivo* behavior of TNF- α and Au in blood differed, with Au-bound TNF- α exhibiting a steeper decay in blood when compared with Au (Figure 1). For example, the concentration of TNF- α in blood decreased from 1.3 ± 0.05 $\mu\text{g/ml}$ measured at 5 min after injection to 0.12 ± 0.02 $\mu\text{g/ml}$ at 4 h, a reduction of approximately 90%. In the same interval, the concentration of Au in the circulation decreased by only 40% (from 68.9 ± 18.9 to 44.9 ± 16.5 $\mu\text{g/ml}$). The concentration of Au in blood later reduced to 12% of the injected value by 12 h and was undetectable after 24 h.

Biodistribution of TNF- α

The concentration of TNF- α in different organs, as measured by ELISA, is plotted in Figure 2A. The relative change in proportion of TNF- α accumulation (average concentration of TNF- α normalized with respect to average concentration of TNF- α at 5 min) has also been plotted in Figure 2B in order to illustrate trends. TNF- α concentration was found to be higher in the liver, spleen, kidney and lung 5 min postinjection presumably owing to higher blood flow in these organs (Figure 2A). Between 5 min and 4 h, the average TNF- α concentration in these organs showed a steep decay similar to blood and was reduced to less than 10% of the initial concentration (Figure 2A & B). On the other hand, average intratumor TNF- α concentration showed a slight increase to 0.53 ± 0.12 ng/g at 1 h from 0.45 ± 0.14 ng/g at 5 min, and remained higher at 4 h (0.25 ± 0.15 ng/g; or ~56% of the concentration at 5 min) relative to the initial concentration (Figure 2A & B). The concentration of TNF- α in the tumor at 4 h was significantly greater than TNF- α present in the liver (0.10 ± 0.02 ng/g) and was comparable to the concentrations measured in the spleen (0.015 ± 0.09 ng/g), lung (0.26 ± 0.06 ng/g) and kidney (0.27 ± 0.12 ng/g). Overall, within the first 4 h following the injection, TNF- α decayed significantly from the blood to less than 10% of the initial concentration and was observed to persist at a higher level in the tumor when compared with the remaining tissues.

Short-term biodistribution of Au

The uptake of Au nanoparticles (normalized with respect to the weight of the tissue) in various organs is plotted in Figure 3A. The relative proportion of Au (average concentration at a time

point normalized with respect to average concentration of Au at 5 min) in these organs has also been plotted for qualitative comparison in Figure 3B. From 0 to 4 h (the interval in which TNF- α decayed significantly in the blood) only the liver, spleen and tumor showed a slight increase in Au concentration (Figure 3A) to approximately two- to four-times the initial value (Figure 3B). After TNF- α decayed in the blood (between 4 and 12 h) a significantly greater accumulation of Au occurred in the liver and spleen with their concentrations measuring 44.4 ± 14.4 and 86.5 ± 39.9 $\mu\text{g/g}$, respectively – an almost eight- to tenfold increase from the value taken initially (Figure 3A & B). The concentration in the tumor tissue increased from 0.5 ± 0.4 $\mu\text{g/g}$ at 5 min to 2.3 ± 0.6 and 4.4 ± 1.8 $\mu\text{g/g}$ at 4 and 12 h, respectively (Figure 3A) – a four- to ninefold increase between 0 and 12 h (Figure 3B). The Au concentrations in the liver, spleen and tumor did not change significantly between 12 h and day 3. In the lung and kidney, the Au levels did not change noticeably between 0 and 4 h but decayed significantly from 12 h onwards. Interestingly, Au was almost undetectable in the lung at day 3 (0.5 ± 0.4 $\mu\text{g/g}$).

Although the liver and spleen showed similar capacity ($\mu\text{g/g}$ tissue) for Au uptake, the majority of the particles were present in the liver due to its greater mass. On a total mass basis (average concentration of Au [$\mu\text{g/g}$] multiplied by the respective tissue weight), the liver and spleen accounted for nearly 45% of the injected dose of Au at 24 h (calculations not shown).

The representative TEM images of the tumor, liver and spleen at day 3 are shown in Figure 4. The particles were noted to be present individually or in small groups (less than five) in the tumor tissue but were detected as big clusters (more than five particles) in the liver and spleen (Figure 5). This is consistent with the higher Au uptake observed in the liver and spleen with AES. Within the cells, the particles were found in membrane-bounded cytoplasmic vesicles.

Comparison of Au distribution in CYT-6091 with Au-PEG

The behavior of Au in blood and its relative uptake in the tumor, spleen and liver between 0 and 24 h is plotted and compared for both CYT-6091 and Au-PEG in Figure 5. Au contained in Au-PEG showed a faster decay in blood when compared with Au in CYT-6091 (Figure 5). In the tumor tissue, Au accumulation from Au-PEG was significantly less when compared with Au in CYT-6091 between 0 and 24 h. On the other hand, the liver showed a much faster accumulation of Au from Au-PEG between 0 and 4 h than CYT-6091, but a similar concentration was measured at 24 h. The concentration of Au in the spleen was also found to be comparable for Au-PEG and CYT-6091 at 24 h. The data suggest that Au-PEG showed a faster decay in blood between 0 and 4 h and a higher relative uptake in the liver in the same interval. However, between 4 and 24 h, Au accumulation was faster in the CYT-6091 group and the concentrations in the liver and spleen became comparable at 24 h for both nanoparticle systems. By contrast, tumor tissue showed a significantly higher uptake of Au from CYT-6091 between 0 and 4 h than from Au-PEG. There was no statistical change in tumor Au concentrations between 4 and 24 h for both the groups.

Long-term biodistribution & elimination of CYT-6091 Au nanoparticles in naive animals

A similar pattern of Au nanoparticle uptake in organs was observed in naive animals as for tumor-burdened animals, with most of the Au nanoparticle uptake occurring in the liver and spleen (Figure 5A). Au uptake in the liver of naive animals peaked at day 1 and remained statistically unchanged up to day 60. After day 60, the Au concentration ($\mu\text{g/g}$ tissue) in the liver dropped from 56.6 ± 6.2 $\mu\text{g/g}$ measured at day 60 to 38.1 ± 2.5 ($p < 0.01$) and 28.5 ± 6.2 $\mu\text{g/g}$ ($p < 0.01$) at days 90 and 120, respectively (Figure 6A). The Au concentration in the spleen (63.9 ± 15.7 $\mu\text{g/g}$ on day 1), however, remained elevated until day 90, and showed a slight reduction (46.3 ± 11.9 $\mu\text{g/g}$) only at day 120 ($p = 0.10$). The relative uptake at day 1 in the lung and kidney was 10- to 20-fold less than the uptake in the liver and spleen, as was also noted in tumor-bearing animals. In the lung, Au was almost undetectable at days 60, 90 and

120. The concentration of Au in the kidney also declined significantly from $9.83 \pm 2.12 \mu\text{g/g}$ after 1 h to $3.04 \pm 0.45 \mu\text{g/g}$ ($p < 0.01$) at day 120. The animals remained healthy for the entire duration of the study (up to 120 days) with no observable signs of fever or toxicity.

The cumulative mass of Au (in μg) accumulated in different tissues (average concentration of Au [$\mu\text{g/g}$ tissue] multiplied by the respective tissue weight) is plotted in Figure 6B. Approximately 53% of the total injected Au could be detected in the liver and spleen at 24 h (similar to tumor-bearing animals, as mentioned previously). Moreover, although the capacity per gram of tissue of Au uptake was similar in the liver and spleen, the liver being larger by weight accumulated the majority of the particles. Approximately 35% ($\sim 43 \mu\text{g}$) of the total injected Au could be detected at day 120 in the liver and spleen. Thus, there seems to be a bimodal pattern of elimination with approximately 47% of the particles undetected after the first 24 h following injection and another 18% lost between days 60 and 120.

Discussion

The current data provide insight on the biodistribution of CYT-6091 after a single intravenous injection in an *in vivo* murine cancer model. The two components of CYT-6091, TNF- α and Au, exhibited different *in vivo* behavior, with bioactive TNF- α sequestering preferentially inside the tumor within the first 4 h after injection. Subsequently, Au was observed to accumulate in the liver and spleen between 4 and 24 h after the loss of TNF- α activity on the particles. Although the liver and spleen exhibited similar capacities per gram of tissue for Au nanoparticle uptake, the liver took up the majority of the nanoparticles due to its larger mass. The longer-term studies in naive animals revealed that the Au accumulated in the liver gradually cleared over time with approximately 35% of the injected Au present in the organs tested at day 120.

Biodistribution of CYT-6091 in tumor-bearing animals

All nanoparticle drug carriers comprise multiple components, mainly a carrier, a therapeutic drug, a hydrophilic component and a targeting ligand. Once in circulation, the biodistribution of each component may vary due to direct metabolism and degradation or indirectly by the absorption of serum proteins that coat the particles in blood [9–11]. Thus, in the case of CYT-6091, it is not unexpected that the pharmacokinetics of the biologic therapeutic, TNF- α , and the particle carrier, Au, differed (Figure 1).

The overall pattern of CYT-6091 distribution can be more suitably described by dividing the data into two time intervals after injection: an early time interval from 0 to 4 h and a late interval from 4 h and beyond. Figure 2 shows that between 0 and 4 h there was preferential accumulation of TNF- α in the tumor tissue compared with the liver and spleen. This is possible by both passive accumulation of nanoparticles through the leaky tumor vasculature due to the enhanced permeability and retention effect as well as by active binding of TNF- α to the cells present in the tumor tissue [1,4]. TNF- α is a vascular modulating agent and studies have shown that it induces a rapid increase in tumor permeability, thus facilitating efflux of more nanoparticles from the circulation into the tumor interstitium [5]. ELISA analysis was performed only up to 4 h, since blood TNF- α concentration during this period declined substantially to less than 10% of the initial value. The majority of the Au uptake in the liver and spleen occurred in the late interval, between 4 and 24 h, suggesting that the presence of active TNF- α on the particles may not only facilitate the delivery of the cytokine to solid tumors but may also prevent the uptake of the nanoparticles by the liver and spleen (Figure 3). The higher concentration of both TNF- α and Au at 5 min in the liver, spleen, kidney and lung after CYT-6091 injection is possibly due to a higher vascular density in these organs relative to the tumor, although this was not verified in the current study.

The role of TNF- α in influencing CYT-6091 distribution is also shown by comparing Au bio-distribution data from CYT-6091 with data from Au-PEG (Figure 5). A faster decay in blood and a higher accumulation in the liver between 0 and 4 h show that the presence of TNF- α reduced CYT-6091 uptake in the liver during this interval. A similar comparison in tumor tissue also suggests tumor-specific binding of TNF- α tagged on Au nanoparticles (Figure 5). The mechanism for this observation is likely due to a difference in the blood-serum interactions after injection, where the presence of both TNF- α and PEG-Thiol possibly alters the absorption of serum proteins, thereby modifying cellular uptake and tissue accumulation [12,13].

The preferential uptake of TNF- α in the tumor tissue is similar to previous data obtained in colon and breast cancer models with CYT-6091, where TNF- α showed an increased half-life and a preferential accumulation in the tumor tissue between 0 and 6 h after injection [4,7]. The persistence of TNF- α for a longer duration in these models could possibly be because of a difference in relative TNF- α -binding efficiency to the tumor cells or due to a difference in density of tumor vascularization. Although the distribution of Au in CYT-6091 was reported for the first time, several other studies with other Au-based nanoparticle systems have reported on the preferential accumulation of nanoparticles (>20 nm) in the liver and spleen as compared with the kidney and lung [14–16]. De Jong *et al.* used 50, 100 and 200 nm Au particles and found that the majority of the injected dose accumulated in the liver at 24 h with little uptake in the brain, kidney and lungs [15]. Similarly, Niidome *et al.* also detected approximately 35% of their injected amount of Au nanorods coated with PEG in the liver in the first 24 h [17].

The TEM images of the tumor, liver and spleen at day 3 (Figure 4) also suggest a difference in the mechanism of uptake between the tumor tissue and the organs of the reticuloendothelial system. The presence of individual or small clusters (less than five particles) suggests a receptor-mediated mechanism by cells present in the tumor tissue (Figure 2). The presence of big clusters of nanoparticles suggests a phagocytic uptake of Au by the cells present in the liver, although further work needs to be performed to correctly infer this [11].

Longer-term biodistribution & elimination of CYT-6091 Au nanoparticles in naive animals

Data suggested that naive animals also exhibited a preferential uptake in the liver and spleen by day 1, when compared with the lung and kidney (Figure 6). The similarity in the uptake and clearance of the Au nanoparticles between the naive and tumor-bearing animals support that the naive animals served as reasonable surrogates to predict the long-term biodistribution and elimination of the Au nanoparticles in tumor-bearing animals. The inability to detect 47% of the injected Au at day 1 and 65% at day 120 suggest both an early Au elimination and a delayed slower elimination mainly from the liver through the hepatobiliary system. Although a direct quantitative analysis at the cellular level was not performed in the current work, the data are consistent with historical evidence showing that a significant fraction of polymer- and protein-stabilized Au nanoparticles are rapidly taken up in the liver and cleared by the hepatobiliary system [18,19]. In a study with polyvinylpyrrolidone-coated 17 nm Au nanoparticles, Hardonk *et al.* showed the particles are taken up by both hepatocytes and Kupffer cells within the first 2–6 h and are secreted earlier (in the first 24 h) from the hepatocytes through a hepatobiliary pathway, and later (after 24 h) through the Kupffer cells via an unknown mechanism. Renaud *et al.* also hypothesized two phases (an early phase and a late phase) of Au clearance from the liver, with the clearance kinetics depending on the initial distribution of the Au complexes between parenchymal and nonparenchymal cells [18].

The present study substantially furthers our understanding of the relative uptake of CYT-6091, and its two major constituents, TNF- α and Au, among different tissues. Bioactive TNF- α from CYT-6091 is preferentially taken up by the tumor, with subsequent accumulation of Au in the liver and spleen after TNF- α bioactivity drops in blood. An important finding on the drug's pharmacokinetics was the elucidation of the relative effects of the drug-carrier components,

and the contribution of both the PEG-Thiol and TNF- α components to the prolonged circulation of the nanoparticle drug. The data also provided evidence of TNF- α binding to the tumor tissue. Overall, CYT-6091 exhibited significant improvements in the targeted delivery of TNF- α to the tumor tissue. The improvements included enhanced and selective uptake of TNF- α by the tumor tissue, and the absence of observable side effects from either the therapeutic drug or the carrier. Future studies will be carried out to identify the cells in the tumor, liver and spleen that take up the particles, and to further elucidate the mechanisms of binding and clearance of CYT-6091. The findings from this study are important for the advancement of Au as a drug-delivery agent and for improvement of the design of nanostructures for *in vivo* biomedical therapeutic applications.

Executive summary

- In the present study, we investigated the biodistribution of CYT-6091 and its constituents, TNF- α and gold (Au), following a single intravenous injection of CYT-6091 in tumor-bearing animals by ELISA and atomic emission spectroscopy. Longer-term clearance of Au up to 120 days was investigated in naive animals.
- The two components of CYT-6091, TNF- α and Au, exhibited different *in vivo* behavior, with active TNF- α sequestering preferentially inside the tumor within the first 4 h after injection. Au was observed to accumulate in the liver and spleen primarily between 4 and 24 h after the loss of TNF- α activity on the particles.
- Both the liver and spleen exhibited similar capacity per gram of tissue for Au nanoparticle uptake, although the liver took up the majority of the nanoparticles owing to its larger size.
- The amount of Au in the liver gradually decreased over the duration of the study. Only 35% of the injected Au was present in the organs tested at 4 months.
- Overall, CYT-6091 exhibited enhanced and selective uptake of TNF- α by the tumor tissue and there was an absence of side effects from either the therapeutic drug or the carrier.
- The current study improved our understanding of the relative effects of the drug-carrier components, and the importance and relative contribution of both the PEG-Thiol and TNF- α components to the prolonged circulation of CYT-6091.

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Figure 1. The biodistribution of CYT-6091 in blood expressed as the average $\pm$ standard deviation of the two components, gold ($\mu\text{g gold/ml}$) and TNF- α ($\mu\text{g TNF-}\alpha/\text{ml}$) ($n = 3\text{--}4$ animals/group)

Au: Gold.

Figure 2. Biodistribution of TNF- α in different organs in tumor-bearing animals after CYT-6091 injection

(A) TNF- α concentration plotted as average $\pm$ standard deviation (ng TNF- α /g tissue). (B) The percent change in average TNF- α concentration in different organs normalized to their respective concentrations 5 min postinjection (n = 3–4 animals/group).

Figure 3. Biodistribution of gold among different organs in tumor-bearing animals after CYT-6091 injection

(A) Au concentration plotted as average $\pm$ standard deviation ($\mu\text{g Au/g tissue}$). (B) The percent change in average Au concentration in different organs normalized to their respective concentrations 5 min postinjection ($n = 3-4$ animals/group).

Au: Gold.

Figure 4. Representative transmission-electron micrographs of the tumor, liver and spleen tissues taken 3 days after the CYT-6091 injection

Gold particles show up individually or as aggregates due to enhanced electron density.

Figure 5. Comparison of gold (Au) concentration average $\pm$ standard deviation ($\mu\text{g Au/g}$ tissue or $\mu\text{g Au/ml}$ blood) in (A) tumor, (B) blood, (C) liver and (D) spleen after Au-poly(ethylene glycol) or CYT-6091 injection in tumor-bearing animals ($n = 3-4$ animals/group)
 Au: Gold; PEG: Poly(ethylene glycol).

Figure 6. Biodistribution of gold among different organs in naive animals

(A) Au concentration plotted as average $\pm$ standard deviation ($\mu\text{g Au/g tissue}$). (B) The total average amount of Au (in μg) present in all organs tested at different time points ($n = 3-4$ animals/group).

Au: Gold.

Table 1

Experimental animal groups (n = 3–4 animals/time point/group).

Group	Animal group	Time points	Analysis
<i>Biodistribution of CYT-6091</i>			
Au	Tumor bearing	5 min, 1, 4, 12 and 24 h, and 3 days	AES and TEM (only 3 days)
TNF- α	Tumor bearing	5 min, and 1 and 4 h	ELISA
<i>Biodistribution of Au-PEG</i>			
Au	Tumor bearing	5 min, and 1, 4 and 24 h	AES
<i>Biodistribution & elimination of CYT-6091</i>			
Au	Naive animals	1 and 24 h, and 60, 90 and 120 days	AES

AES: Atomic emission spectroscopy; Au: Gold; PEG: Poly(ethylene glycol); TEM: Transmission-electron microscopy.