

INTRODUCTION

Currently, the first-line treatment of resectable solid tumors most commonly involves surgery followed by a regimen of chemotherapy and/or radiation. Unfortunately this strategy often fails because of recurrent or metastatic disease. To change this paradigm, new cancer therapies must deliver multifunctional therapeutics to destroy the heterogeneous population of tumor cells present within solid tumors prior to surgical removal. Over the past eight years CytImmune has worked to meet this challenge by developing multifunctional nanoparticles on its patented pegylated colloidal gold (cAu) nanoparticle platform.

The synthesis of these particles was first reported by Michael Faraday, who, in 1857, described the chemical process for the production of nano-sized particles of Au⁰ from gold chloride and sodium citrate. The first medical use of colloidal gold dates back to the 1930's when colloidal gold was used as an effective, first-line treatment of patients with rheumatoid arthritis. In the 1950's the binding of proteins, especially antibodies, directly to colloidal gold particles without altering their bioactivity was key for nano-helical immunodiagnostics and for histology. Today, CytImmune has created a new clinical application for colloidal gold nanoparticles.

The first patented nanodrug developed (designated CYT-6091) actively targets and sequesters recombinant human tumor necrosis factor alpha (TNF) in solid tumors, while avoiding uptake by healthy organs and clearance by the reticuloendothelial system (RES). The drug is comprised of TNF and thiolated polyethylene glycol (PEG-Thiol, an RES avoidance molecule) each of which is individually and covalently bound to the surface of 26 nm cAu nanoparticles.

Our data show that the binding of PEG-Thiol to the surface of the nanoparticle prevents uptake by the liver and spleen, which we hypothesize is due to the ability of each molecule of PEG to become hydrated once in the blood stream. By creating a "water shield" surrounding each particle, the particles do not get opsonized and recognized by the RES (Figure 1), and traffic freely through the circulation.

Our data also show that each molecule of TNF bound to the surface of the pegylated colloidal gold nanoparticles is biologically active. In vivo, our data demonstrate that CYT-6091 accumulates only in solid tumors (Figures 2-3), which we hypothesize, is due to the inherent leakiness of the tumor neovasculature and the presence of TNF binding molecules in and around the tumor. The sequestering of CYT-6091 in the tumor limits the biodistribution of TNF, avoiding healthy organs and tissues, and reducing TNF's known toxicity. Our data also support the hypothesis that once the nanoparticles exit the tumor neovasculature that each molecule of TNF bound to the surface of the pegylated cAu nanoparticles serves one of two functions. First, as expected from TNF's known biological action, CYT-6091 serves as an anti-cancer therapeutic; second and more important, TNF serves as a tumor-targeting ligand bringing 10-times more TNF to the tumor.

Building on this discovery, CytImmune is expanding its repertoire of nanotherapeutics built on the pegylated colloidal gold platform with TNF as a tumor-targeting ligand, by developing new multifunctional therapeutics that may synergize with TNF's anti-cancer action. Previous mouse data and clinical data from a limb-sparing procedure, wherein a tumor-burdened limb is surgically isolated and perfused with high dose TNF, have shown that TNF synergizes with well-known chemotherapeutics. The first of these new nanodrugs, termed CYT-21001 (Figure 4A), is comprised of both TNF and an analog of paclitaxel (Taxol®; Figure 4B) bound to the surface of pegylated colloidal gold nanoparticles. We have demonstrated that this nanodrug delivers 10-times more paclitaxel to solid tumors compared to the paclitaxel analog administered alone, and that the nanodrug causes tumor regression in a TNF-insensitive tumor model.

CYT-6091, which is poised to enter Phase I clinical trials in late 2005/early 2006, is just the first of a family of pegylated colloidal gold-based nanotherapeutics. CYT-21001, the combination of TNF and paclitaxel on a single particle of pegylated colloidal gold, will be the prototype for a rich pipeline of new, patentable cancer nanotherapeutics.

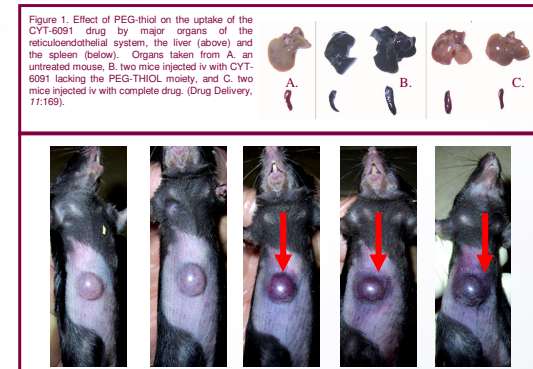


Figure 1. Effect of PEG-thiol on the uptake of the CYT-6091 drug by major organs of the reticuloendothelial system, the liver (above) and the spleen (below). Organs taken from A, an untreated mouse; B, two mice perfused with CYT-6091 lacking the PEG-THIOL moiety, and C, two mice injected iv with complete drug. (Drug Delivery, 11:169).

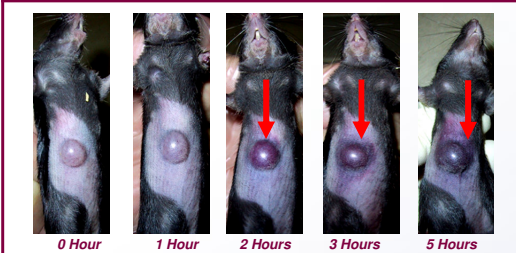


Figure 2. Trafficking of intravenously injected TNF-pegylated colloidal gold (CYT-6091) to the tumor in a mouse. Time indicates hours after a single injection of CYT-6091.

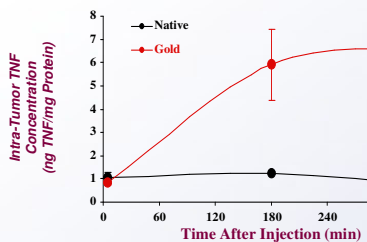


Figure 3. Intra-tumor levels of TNF following injection of 15 mg/mouse of TNF-pegylated cAu or native TNF. Mean ± SEM; n=3/group time point.

METHODS

Preparation of Colloidal Gold Sols: Colloidal gold was produced by the reduction of chloroauric acid (Au³⁺; HAuCl₄) to neutral gold (Au⁰) by sodium citrate using the method described by Horstberger et al., (Biol. Cellulare 36:253, 1979). Briefly, a 4% gold chloride solution (23.03 % stock; Umcorp, South Plainfield, NJ) and a 1% sodium citrate solution (wt/wt; Sigma Chemical Company; St Louis MO) were made in de-ionized water (DIH₂O). 20 ml of the gold chloride solution was added to 5 L of DIH₂O. The solution was vigorously stirred and brought to a rolling boil under reflux. The formation of 26 nm colloidal gold particles was initiated by the addition of 300 ml of the citrate solution, which initiates a series of reduction reactions characterized by changes in the color of the solution to cherry red. The sol was cooled to room temperature and filter through a 0.22µm cellulose nitrate filter and stored at 4°C. Particle size was determined using a dialyzer.

Synthesis of C2-Linked Thiolated Paclitaxel: It is well known that free thiol groups bind to the surface of gold nanoparticles by forming very strong dative covalent bonds. Given that native paclitaxel lacks any thiol groups it was not surprising that it bound poorly to the gold nanoparticles. Thus a thiolated paclitaxel analog was generated and tested for its ability to bind to the colloidal gold nanoparticles. Following the work of Vrudhula et al. (Bio-org. Chem. Lett. 12:3591, 2002) we prepared the paclitaxel derivative shown in Figure 4B (SIM-284 analog). This analog was shown to bind very strongly to the colloidal gold nanoparticles. We observed that reducing agents such as DTT or cysteamine, break the disulfide bond causing the analog to undergo rapid thiolactonization to form paclitaxel.

Saturation Binding Studies: Developing CYT-21001 on the colloidal gold nanoparticle delivery system requires that each component be present in the appropriate concentration to fulfill its function. Thus we performed saturation-binding studies to gauge the capacity of the colloidal gold nanoparticles to bind TNF (kindly supplied by Boehringer Ingelheim Austria, GmbH) and the paclitaxel analog individually and in combination. (NOTE: The characteristics of binding TNF to the colloidal gold nanoparticles were previously described.) For the first study increasing amounts of the SIM-284 analog were added to 1 ml of colloidal gold and allowed to incubate for 15 minutes. The samples were centrifuged and the supernatant and colloidal gold pellet were collected and transferred to tubes containing 10 µg/ml of DTT (to induce the generation of paclitaxel from the analog). Both supernatant and colloidal gold pellets were analyzed for paclitaxel content by EIA. As shown in Figure 5A, at relatively low binding concentrations virtually all of the analog is bound to the colloidal gold particles. As the particles surface become saturated with the analog, the binding to the particles decreases and more of the analog is measured in the supernatant. These data show that the particles have a finite number of binding sites for the SIM-284 analog that will need to be shared with TNF in generating the CYT-21001 vector. To study this relationship we examined the effect of changing the SIM-284 binding concentration on the binding of TNF to the CYT-21001 vector. For this study increasing amounts (0.15 to 20 µg) of SIM-284 analog were added to a solution containing a fixed amount of TNF. These solutions were added to a fixed volume colloidal gold particles and allowed to bind for 15 minutes. After binding the samples were centrifuged at 2,500 rpm for 15 minutes. Supernatant and pellet samples were analyzed for TNF content. As expected as the concentration of the analog increased less TNF bound to the gold nanoparticles (Figure 5B).

Biologic Activity: The SIM-284 analog was evaluated for its biologic activity. For this assay B16/F10 melanoma cells were incubated with increasing concentrations native paclitaxel, or the SIM-284 analog. At the end of the study the cells were harvested and counted in a Coulter Counter. Figure 6 demonstrates that biologically active paclitaxel was generated from the SIM-284 analog after incubation with DTT.

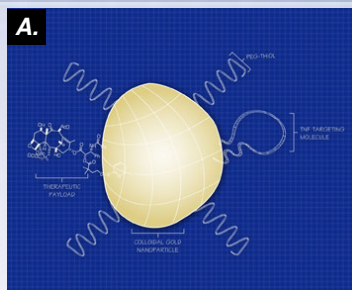
Manufacturing of CYT-21001 for in vivo studies: In this process 0.5, 2, 5 and 15 µg of TNF, SIM-284 analog, and PEG-THIOL, respectively were bound per ml of colloidal gold sol. The binding process is conducted on 1.6 L of colloidal gold sol and involves the simultaneous binding of TNF, SIM-284, and PEG-THIOL with the colloidal gold nanoparticles. (NOTE: Although TNF, SIM-284-analog, and PEG-THIOL are added simultaneously to the nanoparticles, our data show that each reagent binds independently to the nanoparticles (i.e., no cross-linking of the agents occurs). After binding, the solution was concentrated by ultrafiltration to yield a product with the following specifications: TNF concentration: 10 µg/ml; SIM-284 (Paclitaxel) concentration: 100 µg/ml; PEG-THIOL: Adequate to block RES uptake. The recovery of TNF and the SIM-284-analog throughout the binding process was 70 and 81%, respectively and both reagents were completely bound to the particles.

Tumor Uptake Studies: 500 µl of CYT-21001 was injected (IV) into B16/F10 tumor burdened C57BL/6 mice. Control animals received the same dose of TNF and SIM-284 analog diluted in binding buffer. Animals were sacrificed at 3 and 5 hours post injection, and blood and tumor samples were collected and frozen at -80°C. The physical localization of the colloidal gold vector closely resembled the gold localization observed for CYT-6091 (Figure 2). The tumor samples were homogenized and gently centrifuged to remove debris. The tumor homogenates were treated with 100 µg/ml of DTT prior to analysis. The tumor levels of both TNF and paclitaxel were determined by EIA and were normalized to total protein. CYT-21001 vector increased the circulatory half life of TNF and the SIM-284 analog (data not shown). In addition as shown in Figure 7 CYT-21001 sequestered 10 times the amount of TNF and paclitaxel when compared to native TNF/SIM-284 treatment.

TNF: A Tumor Targeting Ligand? Our next objective was to demonstrate a definitive role for TNF as a tumor targeting ligand. For these studies two forms of CYT-20001 were generated. The difference between the two nanoparticle vectors is that one of the vectors (CYT-21001A) lacked TNF, the putative tumor targeting ligand. After manufacturing, equal doses of paclitaxel prodrug were intravenously injected into B16/F10 tumor-burdened C57BL/6 mice. Two hours later the animals were sacrificed, the tumors were harvested, homogenized, and finally treated with DTT to convert the paclitaxel prodrug into paclitaxel. Intra-tumor paclitaxel concentrations were determined by EIA and were corrected for total protein. The data shown in Figure 8 demonstrate that 2 hours after treatment the TNF-targeted vector (CYT-20001) sequestered twice the amount of paclitaxel in the B16/F10 tumors when compared to the untargeted gold vector (CYT21001A). The data are consistent with the data previously described for other untargeted colloidal vectors which only passively accumulate in solid tumors. We believe that the CYT21001A vector accumulates in the B16/F10 tumors by passive extravasation of the leaky tumor vasculature whereas the CYT-21001 vector uses both passive and active (i.e. TNF receptor mediated) targeting.

In Vivo Generation of Paclitaxel by Cysteamine: The data presented in Figures 7-8 suggest that it may be possible to "trigger" release of native paclitaxel from the CYT-20001 vector once the drug arrives at the tumor site. In the following studies we tested whether this could be accomplished with cysteamine, an approved therapeutic for the treatment of retinopathic cystinosis in children in this study B16/F10 tumor-burdened C57BL/6 mice received an intravenous injection of CYT-21001 or a solution containing identical concentrations of native TNF and SIM-284. Three hours after these injections the mice were divided into groups that received either saline or an intra-tumor injection of either cysteamine (5mg) or saline. Three hours after the cysteamine injection, the mice were sacrificed, and the tumor samples were collected and frozen at -80°C and subsequently homogenized. Tumor samples from mice treated with cysteamine were assayed directly with no further modification whereas tumors harvested from saline treated animals were homogenized in DTT. All samples were analyzed for paclitaxel and protein content by EIA or Bradford Assay, respectively. The data shown in Figure 9 demonstrate that in vivo treatment with cysteamine is as efficient at generating paclitaxel from CYT-21001 as in vitro treatment with DTT.

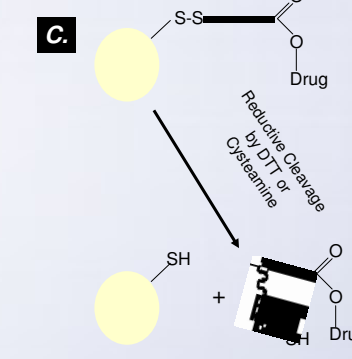
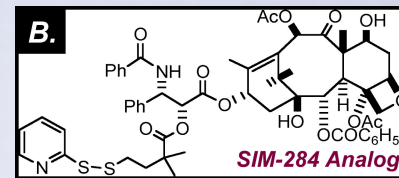
Anti-Tumor Efficacy of CYT-21001: Building on the previous observation we tested the efficacy of the CYT-21001 vector in the B16/F10-C57BL/6 murine tumor model. This study was conducted using the same experimental protocol described above except that tumor volume was monitored after cysteamine induction of paclitaxel release. On the day of the experiment initial tumor measurements were taken and the mice (n=3/group) received an intravenous injection of the native TNF and SIM-284 or CYT-21001. The doses used in the treatments corresponded to an injection of 5µg of TNF and 50 µg of SIM-284 analog. Two hours after this initial treatment paclitaxel generation was triggered by an intra-tumor injection of cysteamine. Control animals received an intra-tumor injection of saline. Tumor volume was monitored over time. As can be seen in the Figure 10, only the combination of CYT-21001 and cysteamine caused reduction and arrest of tumor growth. CYT-21001 without cysteamine treatment did not reduce tumor burden. Finally the inability of native drug treatment, with or without cysteamine treatment, to stop tumor growth is consistent with the marginal delivery achieved without binding to pegylated colloidal gold (Figures 7 and 9).



Figures 4A-C. Figure A. CYT-21001: a TNF targeted paclitaxel prodrug assembled on pegylated colloidal gold nanoparticle.

Figure B. The thiolated self-immolating paclitaxel prodrug.

Figure C. Proposed mechanism of prodrug release of paclitaxel.



RESULTS

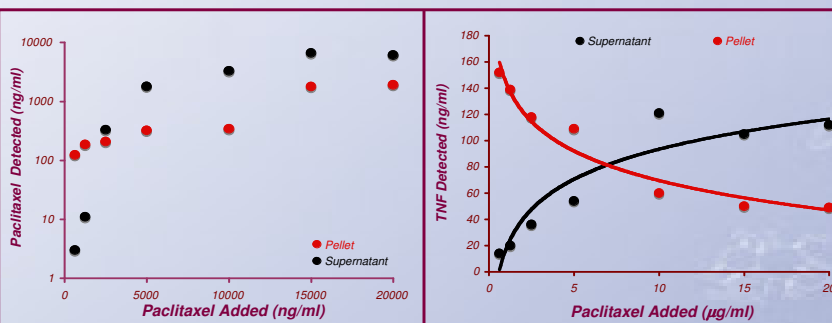


Figure 5. (A) Saturation binding of the SIM-284 analog to colloidal gold nanoparticles. (B) The effect of increasing the amount SIM-284 analog on the binding of TNF to CYT-21001 vector.

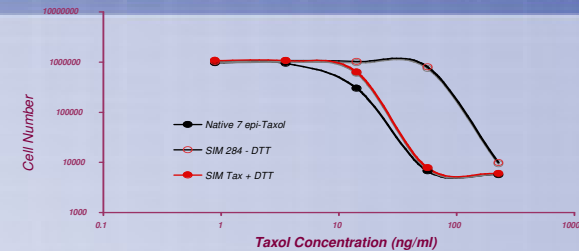


Figure 6. Biologic activity of the paclitaxel SIM284 analog ± DTT pretreatment

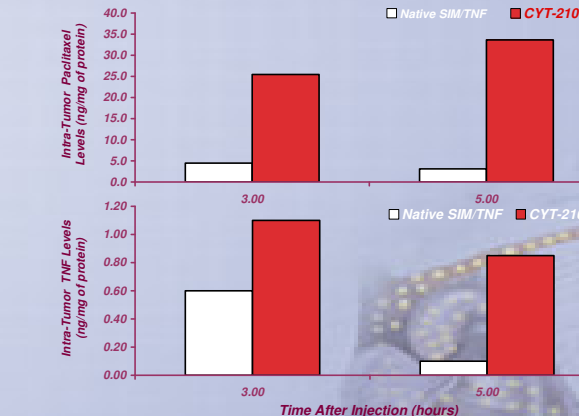


Figure 7. Intra-tumor levels of TNF and paclitaxel following intravenous injection of CYT-21001 or native TNF/SIM-284 into B16/F10 tumor-bearing C57BL/6 mice.

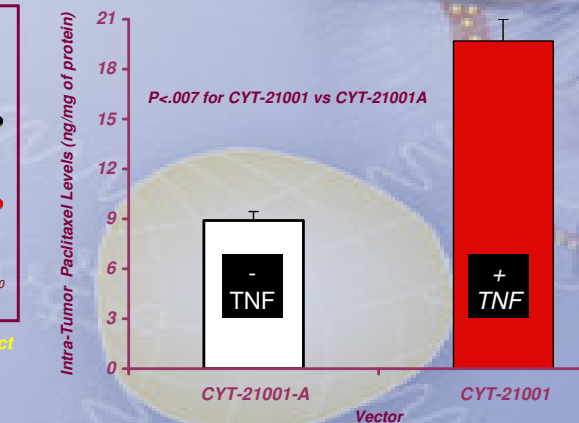


Figure 8. The effect of TNF on the delivery of paclitaxel by CYT-21001

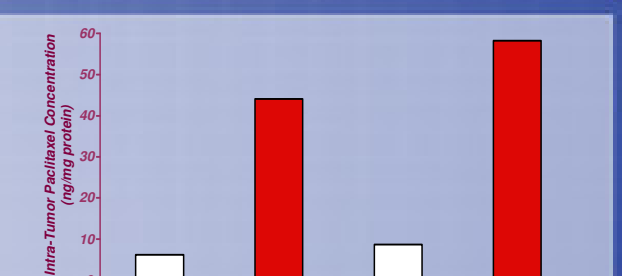


Figure 9. In vivo generation of paclitaxel from CYT-21001 by cysteamine.

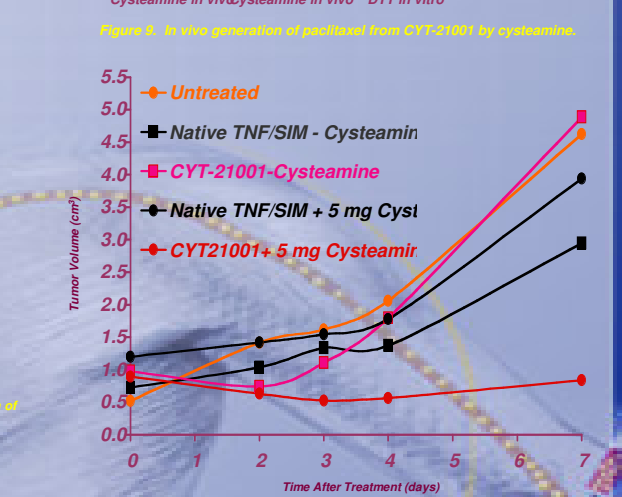


Figure 10. Anti-Tumor Efficacy of CYT-21001 in B16/F10 tumor burdened C57BL/6 mice

SUMMARY

Multiplex, chemically distinct molecules bind to individual colloidal gold nanoparticles.

TNF bound to pegylated colloidal gold nanoparticles acts as an anti-cancer therapeutic and a tumor targeting ligand.

Thiolated forms of small molecule therapeutics, such as paclitaxel, bind directly to the surface of colloidal gold nanoparticles.

Small molecule therapeutics bound in a prodrug form to the surface of pegylated colloidal gold nanoparticles are released as an active drug at the tumor site.

Conclusion

Pegylated colloidal gold nanoparticles are a versatile platform for developing tumor targeted cancer therapies.