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## CYT-21304: A Multifunctional Tumor Targeted Nanotherapy.

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### Introduction

Currently, the first-line treatment of respectable 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 nanotherapies on its patented pegylated colloidal gold (cAu) nanoparticle platform.

Our efforts to address this challenge was to develop a nanomedicine that exploits the biology of solid tumors (i.e., leaky vasculature) to target two active pharmaceutical ingredients, recombinant human TNF and paclitaxel, to solid tumors (Figure 1). CYT-21304 consists of the following:

- cAu particle (The size of 27 nm limits the nanodrug's distribution to the solid tumor due to the unique leakiness of the tumor vasculature.)
- PEG-THIOL (Shields the nanotherapy from clearance by the reticuloendothelial system.)
- TNF (Acts as a targeting ligand and anti-tumor therapeutic.)
- Paclitaxel prodrug (Designed to undergo hydrolysis and generate paclitaxel upon its delivery to the tumor.)

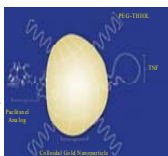


Figure 1. Blueprint for CYT-21304

### Methods

#### Binding to Gold

The thiolated paclitaxel Analog 13 was synthesized by the Kingston group at Virginia Polytechnic Institute and State University. The synthesis will be described in an upcoming publication. The analog was bound to the cAu nanoparticles following the methods described in Paciotti et. al. (Drug Development Research, 67:47-54, 2006) and Paciotti et. al. (Drug Delivery, 11:169-183, 2004).

#### Paclitaxel Quantitation

Conversion of analog was determined by RP-HPLC (Proto 200, Higgins Analytical, Inc.) under standard HPLC conditions. The forced conversion of soluble or cAu bound analog was achieved by incubation in hydrolysis buffer (NaHCO<sub>3</sub> and DTT). In certain studies, paclitaxel content was determined by paclitaxel specific ELISA (Hawaii Biotech).

#### Cell Proliferation Assay

The potency of the soluble and cAu bound drug was determined in an in vitro bioassay using the A2780 indicator cell line. The potency of these drugs was compared to soluble paclitaxel.

#### Pharmacokinetic and Tumor Uptake Studies

- C57BL/6 mice were implanted with B16-F10 melanoma cells. When tumors reached approximately 500 mm<sup>3</sup> the mice received a single intravenous injection of either soluble paclitaxel, CYT-21304, or soluble analog at a dose of 2.5 µg per mouse.
- At the indicated time points, mice (n=3/group/time point/ formulation) were sacrificed and whole blood and tumors collected.
- Tumors were homogenized in TBS.
- Each sample was analyzed under two conditions:
  - No modification to measure hydrolyzed drug.
  - Sample was subjected to hydrolysis buffer to measure total paclitaxel content.
- Tumor concentrations were corrected for total protein.

#### Efficacy Studies

C57BL/6 tumored mice received an intravenous injection of either soluble paclitaxel at 2.5 or 40 mg/kg or CYT-21304 at 2.5 mg/kg on day 0, 5 and 7, and tumor volume was measured over time.

### Results

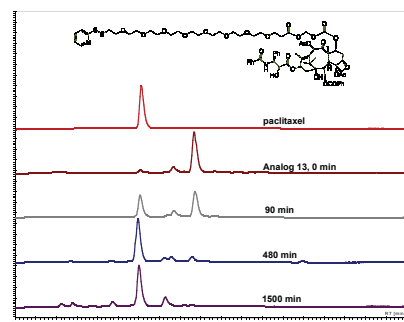


Figure 2. Forced conversion of analog 13 to paclitaxel. One mg of analog 13 was diluted in 1 ml of solution containing dithiothreitol (DTT) to induce self-irradiation, and NaHCO<sub>3</sub> to induce hydrolysis. Samples were incubated for indicated times and analyzed by RP-HPLC.

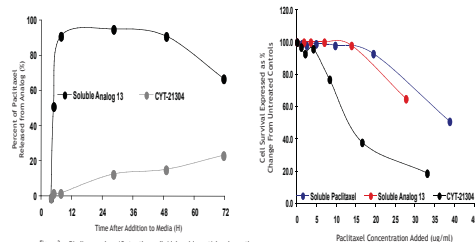


Figure 3. Binding analog 13 to the colloidal gold particle slows the hydrolysis of the analog to paclitaxel. Lipidated material was reconstituted with DMSO and equal amounts of either the soluble analog or its cAu bound variant were spiked into tissue culture media and analyzed over a 72 hour period. Amount generated is compared to the total amount of paclitaxel measured following forced conversion by incubation in a buffer containing dithiothreitol (DTT), to induce self-irradiation, and NaHCO<sub>3</sub> to induce hydrolysis.

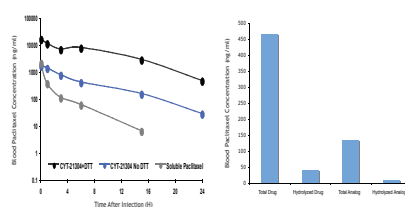


Figure 5. The circulatory half-life of CYT-21304, and the corresponding paclitaxel generated from the nanodrug, are significantly longer than soluble paclitaxel and analog 13. B16-F10 tumor-bearing C57BL/6 mice (30 mice/group) were intravenously injected with 50 µg of paclitaxel (dissolved in the Cremaphor EL diluent), the soluble analog or the same dose of CYT-21304. At selected time points after injection the mice were bled through the retro-orbital sinus. Prior to analysis the samples were divided into 2 equal aliquots. One aliquot was measured directly in the assay without any modification. This analysis provided us with a quantitative assessment of the colloidal gold bound analog undergoing hydrolysis in the blood. The second sample was treated with the hydrolysis buffer (DTT, NaHCO<sub>3</sub>) to gauge the total amount of drug present in the blood. The blood was analyzed for paclitaxel concentration by ELISA.

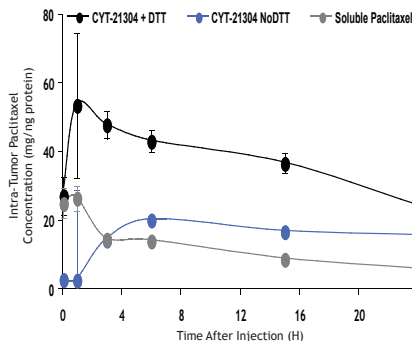


Figure 6. CYT-21304 actively sequesters both the analog and paclitaxel in solid tumors. Tumor samples were harvested from the animals of the pharmacokinetic study and were flash frozen, and then subsequently homogenized in R65 using a polytron tissue disruptor. Solids were removed by allowing the homogenate to stand on ice for 20 minutes. The resultant supernatant was analyzed for paclitaxel concentration, by ELISA, and for total protein using a commercial protein assay (BioRad, Hercules, CA, USA).

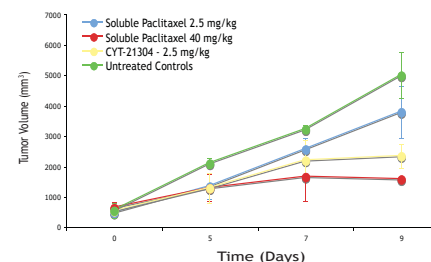


Figure 7. Anti-tumor efficacy of soluble paclitaxel or CYT-21304. C57BL/6 mice were implanted with B16/F10 melanoma cells. Once the cells formed tumors with an approximate volume of 500 mm<sup>3</sup> the mice (n=4/group) received an injection of either soluble paclitaxel or the CYT-21304 formulation. Soluble treatments were given at a dose of 2.5 mg/kg and 40 mg/kg, whereas CYT-21304 were administered at a dose of 2.5 mg/kg. The mice were treated on day 0, 5 and 7, and tumor responses were determined by measuring tumor volume during the course of the study.

### Summary and Conclusions:

- CYT-21304 is a novel nanotherapy that delivers two active pharmaceutical ingredient, TNF and paclitaxel, to solid tumors to produce a potent anti-tumor effect.
- CYT-21304 increases the circulatory half-life of paclitaxel and analog when compared to soluble analog and soluble paclitaxel.
- CYT-21304 actively sequesters analog and paclitaxel in solid tumor when compared to soluble paclitaxel.
- CYT-21304 is an effective anti-tumor agent at a lower dose compared to soluble paclitaxel.