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Research Overview

Colloidal Gold Nanoparticles: A Novel Nanoparticle Platform for Developing Multifunctional Tumor-Targeted Drug Delivery Vectors

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Nanotechnology applied to biological problems represents an emerging field with the potential to offer extremely sensitive diagnostics and targeted cancer therapies. However, to achieve these goals, nanoparticle delivery systems must outwit the many barriers that are intrinsic to the body's defenses, as well as those that develop during the growth and progression of tumors. The science is advancing and, for example, true nanoscale tumor-targeted drug delivery vectors are now able to reduce the likelihood of opsonization in the bloodstream and uptake by the reticuloendothelial system. Other advances hold promise for delivering multiple therapeutic agents to non-homogeneous populations of cancer cells in solid tumors. We briefly summarize herein our attempts to build such multifunctional nanotherapeutics using colloidal gold nanoparticles. Specifically we discuss the development of colloidal gold-based drugs that are designed to target the delivery of TNF and paclitaxel to solid tumors. Drug Dev. Res. 67:47–54, 2006.

Key words: colloidal gold; nanotechnology; nanoparticles; drug delivery; targeting; therapeutics; diagnostics; cancer; TNF; paclitaxel; opsonization

INTRODUCTION

The emerging field of bionanotechnology (or nanobiotechnology) offers the potential for the development of exquisitely sensitive diagnostics and organ/tumor-targeted therapies. For example, the miniaturization of diagnostics may not only provide clinicians with a more complete snapshot of blood chemistries, hormones, and growth factors in both normal and diseases states, but may also allow them to track the efficacy of putative therapeutics [Koehne et al., 2004]. Complementing its diagnostic advances, bionanotechnology also holds the promise of increasing the therapeutic index, a measure of the benefit/risk ratio, of current cancer therapies, as a prime example [Papisov, 1998; Moghimi and Patel, 1998; Woodle, 1998; Nafayasu et al., 1999; Maruyama et al., 1999]. Indeed, the blending of material science and tumor biology is leading to the development of innovative vectors with the potential of achieving the long-sought-after goal of tumor-targeted drug delivery, getting the active agent(s) solely where it's needed, at the solid tumor. Yet, to successfully achieve this goal, nanoparticle delivery systems must overcome the biological barriers (for a synopsis, see Table 1) that are naturally

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TABLE 1. Strategies for Overcoming Biological Barriers to Nanoparticle-Based Cancer Therepies

Barrier	Technology	Reference
1. Clearance by the reticuloendothelial system (RES) by opsonization	• Hydrophilic polymers either coating or bound to the nanoparticle. • Smaller nanoparticles	Baban and Seymour [1998]; Redhead et al. [2001]; Moghimi et al. [1991]
2. Clearance by the RES by size	• Smaller nanoparticles	Chen and Weiss [1973]
3. Tumor angiogenesis leading to an increase in interstitial (tumor) fluid pressure	• Passive accumulation of particles through extravasation of the leaky tumor vasculature • Vascular normalization • Cytokine/chemotherapy-mediated reduction of interstitial fluid pressure (IFP)	Molema et al. [1997]; Tong et al. [2003]; Jain [2005]; Kristensen et al. [1996]; Nedrebo et al. [1999]
4. Ligand/receptor-based nanotherapeutic targeting	• Incorporation of a tumor targeting ligand and a therapeutic into the nanoparticle	Cristiano and Roth [1996]; Gottschalk et al. [1994]; Singh [1999]; Curnis et al. [2002]; Tuffin et al. [2005]; Sachdeva [1998]
5. Barriers within the tumor interstitium: intra-tumor barriers established during the formation of the tumor extracellular matrix	• Using true ''nanometer''-sized particle delivery systems	Pluen et al. [2001]
6. Cellular heterogeneity of solid tumors	• Multifunctional nanotherapeutics	Spremulli and Dexter [1983]; Dexter et al. [1978]

present in the body, as well as those that develop during tumor growth and progression.

Having learned from the hard lessons of past research, bionanotechnologists now have available effective strategies to engineer nanoparticle delivery systems to address the first five barriers listed in Table 1. For example, tumor-targeting drug delivery vectors are now approaching "true" nanometer size, which will not only diminish the likelihood of their being opsonized in the blood and taken up by the reticuloendothelial system (RES; i.e., larger particles activate complement better than smaller particles) but also prevent their clearance in the narrow confines of the inter-endothelial slits present in the red-pulp of the spleen. To further improve RES avoidance, hydrophilic polymers may be grafted onto the surface of these nanoparticle systems. Once these nanoparticle vectors are free to circulate throughout the body, they may passively as well as actively sequester in and around a solid tumor due to the inherent leakiness of the tumor neovasculature and the presence of tumor-specific ligands on the surface of these nanoparticle vectors.

The last element in building these nanotherapeutics lies in the ability to develop vectors that effectively deliver multiple therapeutic agents to the heterogeneous populations of cancer cells comprising a solid tumor. In its simplest model, a solid tumor may be viewed as an organ containing multiple cell types that act in concert to promote tumor growth [Spremulli and Dexter, 1983, Dexter et al., 1978]. Thus, drugs that target a single type of cell for therapeutic intervention may only provide marginal anti-tumor effect. Furthermore, in many cases solid tumor cells exhibit a continuum of phenotypes during disease progression and/or in response to therapy. Consequently, it seems unlikely that single agent therapies, regardless of the ability of the nanoparticle delivery system to sequester them in the solid tumor, will prove effective against the myriad cells present within the malignancy. To overcome this limitation, next generation nanotherapeutics must not only find their way to the solid tumor but must also effectively destroy the diverse populations of cells promoting tumor growth. In the following sections, we review our attempts to build such multifunctional nanotherapeutics using colloidal gold nanoparticles. Specifically, we will discuss the development of two colloidal gold-based drugs that are designed to target the delivery of TNF or the combination of TNF and paclitaxel to solid tumors.

A PRIMER ON THE USE OF COLLOIDAL GOLD NANOPARTICLES IN BIOTECHNOLOGY

The generation of colloidal gold nanoparticles was first described by Michael Faraday in 1857 when he described the synthesis of multicolored solutions (Fig. 1) by reacting gold chloride with sodium citrate [Faraday, 1857]. Unbeknownst to him, what he actually described was the synthesis of colloidal gold nanoparticles that ranged from 12–60 nm in diameter. Since that time, gold nanoparticles have been used to meet a variety of needs in science and medicine. In the 1950s, the discovery that the particles bound protein biologics

without altering their activity paved the way for their use in hand-held immunodiagnostics and in histopathology [Chandler et al., 2001]. More recently gold nanoparticles have been assembled into scaffolds for use in deoxyribonucleic acid (DNA) diagnostics and biosensors [Mirkin et al., 1996].

Of particular relevance to the current communication was the long-standing use of colloidal gold in the treatment of rheumatoid arthritis and as well as the use of radioactive gold nanoparticles to treat liver cancer [Rubin and Levitt, 1964, Root et al., 1954]. These historical data, combined with the data from our own Good Laboratory Practices (GLP) toxicology study in rabbits (data not shown), suggest that colloidal gold nanoparticles are relatively inert and biologically compatible carriers.

Nearly 50 years after their description as antineoplastic agents, colloidal gold nanoparticles are reemerging as lead candidates in the field of tumor-targeted nanotherapy. For example, Hirsch et al. [2003] recently described the use of colloidal gold nanoshells for the thermal ablation therapy of solid tumors. They described the synthesis of a gold/silica nanoshell comprised of an aminated silica core particle, which was studded with ultra-small (1–3 nm) gold particles. Exposing these particles to a light source (i.e., diode laser) with a wavelength of 820 nm caused the electrons present on the gold surface (i.e., plasmons) to become excited, resulting in particle heating. Interstitial injection of these particles near the tumor followed by laser light excitation caused a significant reduction of transmissible venereal tumors (TVTs) growing in severe combined immunodeficiency disease (SCID) mice.

Over the past 5 years, our lab has focused on developing a tumor-targeted drug delivery system based on a pegylated colloidal gold (cAu) nanoparticle platform. In the following sections, we present the rationale for developing these nanotherapeutics and the proof of concept data demonstrating the utility of the colloidal gold nanoparticle platform for developing multifunctional nanotherapeutics.

ISOLATED LIMB PERFUSION PROCEDURE: A SURGICAL METHOD OF TUMOR-TARGETED DRUG DELIVERY

In 1975, Carswell and colleagues isolated a factor from the serum of endotoxin-treated mice that caused the hemorrhagic necrosis of solid tumors [Carswell et al., 1975]. Based on these exciting preclinical data, this factor, later termed Tumor Necrosis Factor (TNF) alpha, was rapidly developed for clinical testing in cancer patients. However, by the mid-1980s hopes of using TNF as a cancer therapy were nearly dashed by the life-threatening toxicities it induced [Alexander et al., 2000], a fate shared by many cytokines that showed promise in pre-clinical tumor models. Yet the pioneering work of Lienard and Lejuene [Lejeune 1995, Lienard et al., 1992] breathed new life into the therapeutic uses of TNF, as they demonstrated vast improvements in the therapeutic index of TNF by surgically limiting its delivery to solid tumors. For TNF, the isolated limb/organ procedure (ILP) demonstrated that the combination of surgically localized delivery of the cytokine with systemically administered chemotherapies induced sustainable anti-tumor responses in patients failing traditional standards of care. In effect, by localizing the delivery of TNF to solid tumors, Lienard and Lejuene harnessed the biology of TNF to induce at least one of the possible mechanisms by which the cytokine induces an anti-tumor response. For example, TNF has been shown to cause apoptosis directly in various tumor cell types [Carswell et al., 1975; Helson et al., 1975], to activate and drive immune-based anti-tumor responses [Asami et al., 1989], and to induce vascular leak in tumor blood vessels [Nawroth and Stern, 1986; Brett et al., 1989], leading to a destruction of the interstitial fluid pressure (IFP) gradient in solid tumors [Kristensen et al., 1996; Nedrebo et al., 1999]. Thus, the rational design of nanotherapeutics that simulate this surgical procedure may prove to be an effective strategy to developing new tumor-targeted therapies. Specifically, our goal was to develop a colloidal gold-based nanotherapeutic that targeted the delivery of a biologic, TNF alpha, alone or in combination with a chemotherapeutic, to solid tumors.

DEVELOPING MULTIFUNCTIONAL NANOTHERAPEUTICS ON A COLLOIDAL GOLD NANOPARTICLE PLATFORM

CYT-6091 is a multivalent drug that is assembled on 26-nm particles of colloidal gold and designed to actively sequester recombinant human TNF within solid tumors. The drug is manufactured by covalently linking molecules of TNF and thiol-derivatized polyethylene glycol (PEG-THIOL) onto the surface of the colloidal gold nanoparticles [Paciotti et al., 2004]. The PEG-THIOL and TNF do not bind to each other (i.e., crosslink together to make pegylated TNF). Rather, each of these molecules binds separately to the colloidal gold surface, resulting in TNF being bound to pegylated colloidal gold nanoparticles. Each component of the drug serves a specific function in achieving tumor-specific drug delivery. First, the PEG-THIOL serves to hydrate the nanoparticles, and in so doing shields the nanoparticle drug from detection and clearance by the RES (Fig. 2A–C). As shown in Figure 2B, colloidal gold vectors lacking PEG-THIOL are

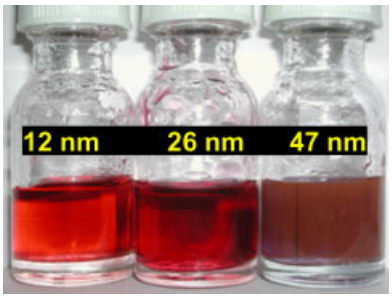

Fig. 1. Colloidal gold nanoparticles.

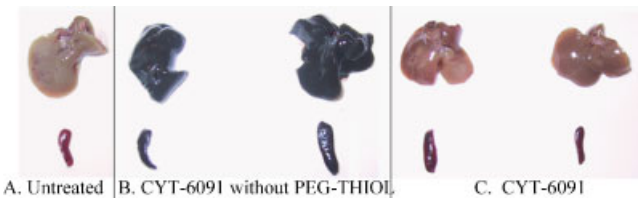

Fig. 2. Effect of PEG-THIOL on the uptake of the CYT-6091 by the RES (i.e., the liver and spleen) in MC-38 tumor-burdened C57/BL6 mice. Reproduced from [Paciotti et al., 2004] with permission of the publisher.

rapidly taken up by the liver and spleen and the gold nanoparticles aggregate to form black precipitates in these organs. In contrast, vectors containing PEG-THIOL avoid immune detection and are not taken up by the liver and spleen (Fig. 2C).

Second, TNF not only serves as the therapeutic responsible for the anti-tumor effects, but also serves as a ligand that targets the nanoparticle drug specifically to a solid tumor (Fig. 3A). As shown in Figure 3A, the tumor sequestration of CYT-6091 is easily documented as the MC-38 solid tumors acquire the reddish-purple color (red arrows) of the colloidal gold nanoparticles. The end result is a 7–10-fold increase in the amount of TNF that is delivered to the tumor when it is bound to pegylated colloidal gold nanoparticles (Fig. 3B). The pattern of TNF accumulation caused by CYT-6091 is specific to the tumors since TNF concentrations in healthy organs decreased over the same time period [Paciotti et al., 2004] (Fig. 4).

The ability to concentrate TNF within the MC-38 tumors improved the efficacy of a given dose of TNF. These data show that 7.5 μg of CYT-6091 was as effective as 15 μg of native TNF in causing tumor regression. In fact, 25–30% of the animals exhibited a complete response after a single 7.5-μg injection of CYT-6091. Finally, CYT-6091 was also safer than native TNF, since the mortality rates for the 15- and 7.5-μg TNF doses were 33 and 15%, respectively, while in contrast none of the animals receiving CYT-6091 died [Paciotti et al., 2004].

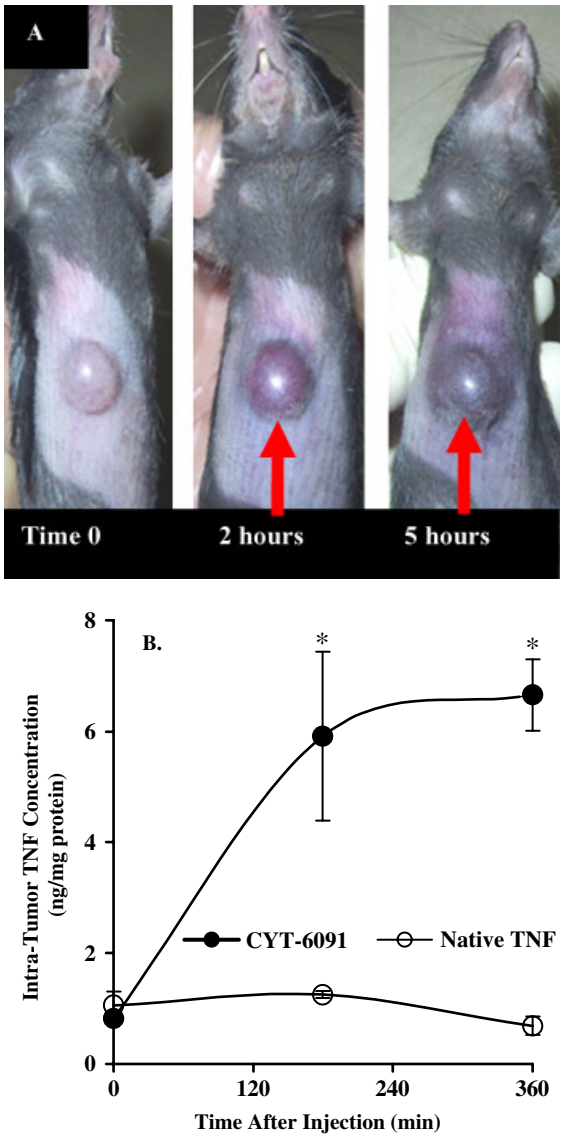

Fig. 3. **A:** Visual documentation of the accumulation of CYT-6091 (red arrows indicate location of the particle drug) in MC-38 tumors. **B:** Sequestration of CYT-6091 and TNF in MC-38-tumors. MC-38 tumor-burdened C57/BL6 mice were injected with 15 μg of either native TNF or CYT-6091. TNF actively accumulates in the MC-38 tumors (*$P<0.05$ vs. native TNF Treatment and the time 0 point for CYT-6091 treatment). Reproduced from Paciotti et al., [2004], with permission of the publisher.

Three main observations were made during the development of the CYT-6091 vector. First, colloidal gold particles can bind more than one molecule (i.e., TNF and PEG-THIOL). Second, PEG-THIOL significantly alters the in vivo distribution of the vector by significantly reducing its uptake and clearance by the RES. And, third, the TNF associated with CYT-6091 acts as both a tumor-targeting ligand and as an anticancer therapeutic.

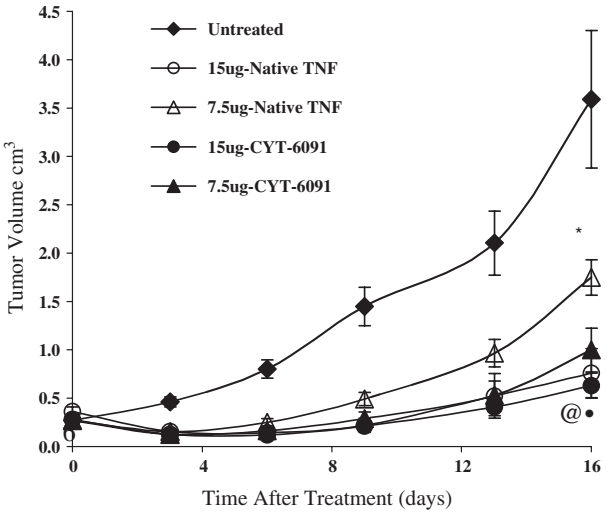

Fig. 4. Comparison of anti-tumor efficacy of native and CYT-6091 in MC-38 tumor-burdened C57/BL6 mice. Note that the percent survival for the native and CYT-6091 at the 15-μg dose was 33 and 100%, respectively. $^{*}P<0.05$ for 7.5-μg dose of Native vs. Untreated controls; $@P<0.05$ for 15-μg dose of Native TNF and CYT-6091 vs. Controls; $P<0.05$ for 7.5-μg dose of CYT-6091 vs. Native TNF. Reproduced from Paciotti et al., 2004, with permission of the publisher.

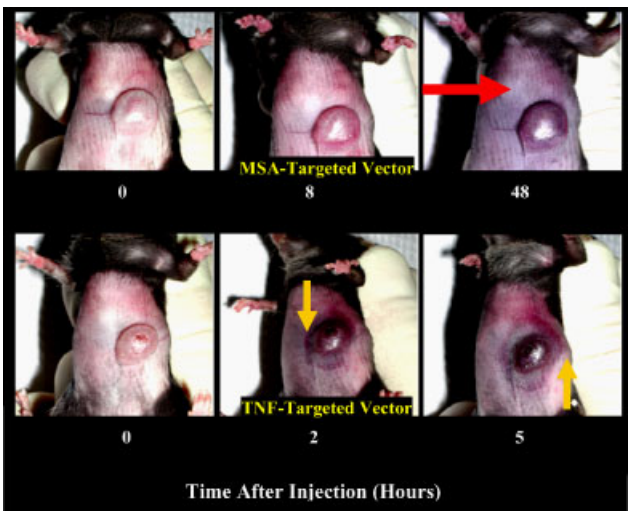

Fig. 5. Targeted/active (TNF) versus passive (MSA) accumulation of the PEG-THIOL colloidal vectors.

To demonstrate the latter concept, we compared the tumor uptake of colloidal gold vectors targeted with either TNF or murine serum albumin (MSA). Subsequent to their manufacture, identical amounts of each vector were injected in MC-38 tumor-burdened mice and the appearance of each vector in the tumor was simply documented by digital photography. As can be seen in Figure 5, by 48 h post-injection, the MSA vector was present in the MC-38 tumor, since the tumors are darkly stained with the color of the colloidal gold vector. In contrast, animals injected with CYT-6091 showed intense and broad tumor staining within 2–3 h after injection.

These data support a dual mechanism for the accumulation of colloidal gold nanoparticles in the tumor. First, the ability of the MSA vector to accumulate within the tumor suggests that PEG-stabilized particles can passively accumulate within the tumor. This mechanism is similar to the mechanism described for the passive accumulation of liposomes within tumors and is mediated by the extravasation of these vectors across the leaky tumor neovasculature. However, the purple coloring of the skin (highlighted by the red arrow in Fig. 5) in the animals treated with the MSA-based vector is really only apparent after 48 h. The second targeting mechanism, an active process, involves the binding of TNF to its receptors present in the tumor interstitium. Thus, upon passive extravasation, the TNF on the gold nanoparticle serves to bind and arrest the nanoparticle within the solid tumor to actively sequester TNF in the solid tumor.

BEHAVIOR OF CYT-6091 IN A TNF-INSENSITIVE TUMOR MODEL: RATIONALE FOR DEVELOPING MULTIFUNCTIONAL THERAPEUTICS ON THE COLLOIDAL GOLD NANOPARTICLE PLATFORM

Although these results were promising, we recognized that the above effects could be attributed to the sensitivity of this (MC-38) tumor cell line to TNF. Thus, the observed accumulation of TNF and anti-tumor action may have been due to a direct action/binding of TNF on these tumor cells. To test this hypothesis, we conducted similar studies of CYT-6091 using a human TNF-resistant murine tumor model. Specifically, C57/BL6 mice implanted with B16/F10 melanoma cells were treated with CYT-6091 as described above. Interestingly, we observed an apparent disconnect between the accumulation of TNF and its efficacy in this model. Although the drug caused tumor-specific uptake and sequestration of TNF, as was observed in the MC-38 tumor model, it only caused transient inhibition of B16/F10 tumor growth. Although in this experimental model, CYT-6091 caused only transient inhibition of tumor growth, the drug did significantly change the physical morphology of the tumors. In brief, 30 min after treating the mice with CYT-6091, the tumors appeared to swell, suggesting edema in and around the tumor area. This swelling, which was not observed in tumor-burdened mice that received the same dose of native TNF, did not resolve itself throughout the observation period.

STRATEGY FOR DEVELOPING MULTIFUNCTIONAL COLLOIDAL GOLD NANOPARTICLE DRUGS: BINDING CHEMICALLY DISTINCT MOIETIES TO THE SAME GOLD NANOPARTICLE

To address this potential limitation of CYT-6091 as a single agent therapy, we are currently focused on developing a colloidal gold nanoparticle that more closely simulates the ILP paradigm. This second vector, termed CYT-21001, is designed to target the delivery of both TNF and a chemotherapy, namely paclitaxel, to solid tumors. Developing the proposed multifunctional vectors on the colloidal gold nanoparticle delivery system requires that each component be present in the appropriate concentration to fulfill its function. As a corollary, the colloidal gold-platform must possess the flexibility to accommodate multiple binding chemistries, since the development of CYT-21001 requires that chemically distinct molecules (i.e., a protein, a polymer, and an organic small molecule) share the same nanoparticle surface.

For CYT-6091, identifying such binding chemistries for TNF and PEG-THIOL was facilitated by the presence of available sulfhydryl groups naturally present on the protein or synthesized on the polymer. However, given the structure of paclitaxel it was not surprising that without modification this small molecule does not bind to colloidal gold nanoparticles. Thus, a series of thiolated paclitaxel analogs (paclitaxel-SH), such as those shown in Figure 6, were generated and interrogated for their ability to bind to colloidal gold nanoparticles and remain biologically active once bound to the particle. Finally, each analog was tested for its compatibility to co-bind with TNF and PEG-THIOL on the same colloidal gold nanoparticle. The presence of both TNF and the thiolated paclitaxel analog was determined using a cross-antibody enzyme immunoassay (EIA) as shown in Figure 7. This qualitative EIA uses a TNF monoclonal antibody to capture the particle-bound TNF present on the TNF/paclitaxel-SH chimeric particle. Subsequently, the thiolated paclitaxel-SH analog is detected with an enzyme-labeled paclitaxel-specific polyclonal antibody.

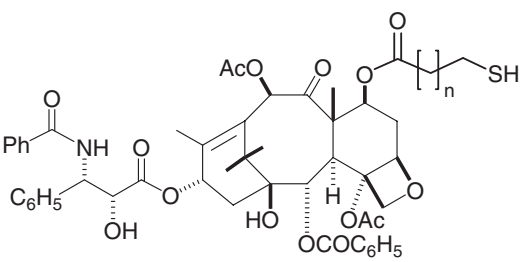

Fig. 6. Thiolated paclitaxel analogs.

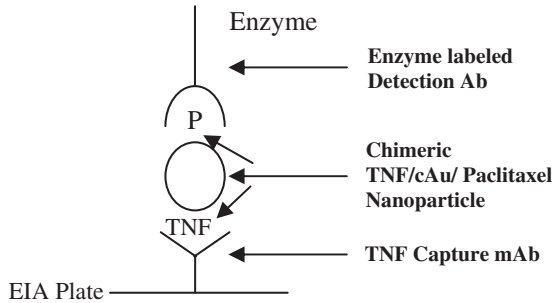

Fig. 7. The cross-antibody EIA.

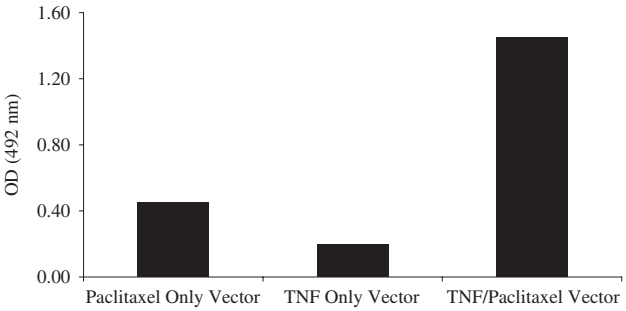

Fig. 8. TNF/cAu/paclitaxel chimera

Control vectors lacked either TNF or paclitaxel. As shown in Figure 8, the nanoparticles bound with both TNF and the paclitaxel-SH analog generated a signal over the control vectors, indicating that both TNF and paclitaxel were bound to the same colloidal gold nanoparticle.

DESIGN OF A PT-CAU-TNF/PACLITAXEL DRUG FOR SITE SPECIFIC RELEASE OF NATIVE PACLITAXEL IN SOLID TUMORS

Although encouraged by our initial results, we also observed that on many occasions the thiolation reaction reduced the biological activity of the paclitaxel analog when compared to the native drug. To address this concern, we developed a thiolated prodrug form of paclitaxel that bound to the colloidal gold nanoparticle and remained inactive until its release from the particle by reductive cleavage. In vitro studies showed that in the presence of a strong reducing agent, such as dithiothreitol (DTT), nearly 100% of the inactive prodrug was converted to active/native paclitaxel (Fig. 9A). More importantly, we observed that this conversion occurred irrespective of whether the analog was free in solution or covalently linked to the colloidal gold particles.

These data were confirmed in an in vitro bioassay in which equimolar amounts of native or prodrug paclitaxel were added to B16/F10 melanoma tumors

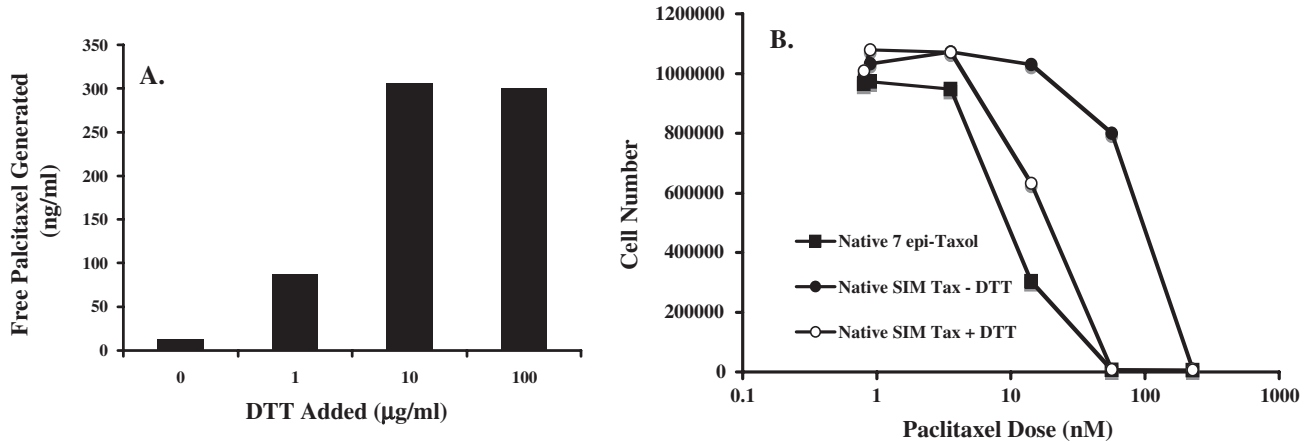

Fig. 9. **A:** Dose-dependent generation of paclitaxel from thiolated paclitaxel prodrug by DTT. **B:** Comparison of the biological activity of the prodrug ± DTT with native paclitaxel.

TABLE 2. Generation of Native Paclitaxel by DTT and the Clinically Approved Reducing Agent Cysteamine

Reductant	Free paclitaxel generated (ng/ml)	Percent of theoretical
DTT	19.50	80
Cysteamine	11.50	50

cells growing in culture. In one group, the paclitaxel prodrug was pretreated with DTT prior to its addition to the cells. (NOTE: The concentration of DTT used did not have any effect on cell growth.) After the addition of the reagents, the cells were cultured for an additional 5 days and cell growth was measured by determining cell number in a Coulter counter. As shown in Figure 9B, the paclitaxel prodrug (closed circles) remained inactive unless it was treated with DTT (open circles).

Recognizing that DTT will not be used in vivo, we tested whether the approved therapeutic cysteamine [Kleta and Gahl, 2004], used for the treatment of retinopathic cystinosis in children, would reduce the paclitaxel prodrug to generate native paclitaxel. This hypothesis, which was initially confirmed in vitro (Table 2), suggested that the agent might be used to trigger the generation of native paclitaxel once the TNF-targeted colloidal gold vector arrives at the tumor site. In vivo testing will determine if this strategy of using cysteamine to reduce the prodrug releases a sufficient amount of paclitaxel to cause tumor regression.

SUMMARY AND FUTURE DIRECTIONS

The central theme of this communication is the use of pegylated colloidal gold nanoparticles as a platform technology for the development of multifunctional tumor targeted nanotherapeutics. The data presented above provide the initial proof of concept for this approach as a means of targeting known anticancer agents to two genetically and phenotypically divergent solid tumors. Future research will identify the role that each therapeutic plays in achieving anti-tumor responses, not only as a means of understanding the drugs' mechanisms of action but to also guide the development of additional multifunctional colloidal gold-based therapies, including those targeted to generate an active immune response within the solid tumor.

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