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A novel intralymphatic nanocarrier delivery system for cisplatin therapy in breast cancer with improved tumor efficacy and lower systemic toxicity in vivo

Mark S. Cohen, M.D., F.A.C.S.[a,†], Shuang Cai, M.S.[b,†], Yumei Xie, Ph.D.[b], M. Laird Forrest, Ph.D.[b,*]

[a]Department of Surgery, University of Kansas, Medical Center, Kansas City, KS, United States; [b]Department of Pharmaceutical Chemistry, 2095 Constant Ave., University of Kansas, Lawrence, KS 66047, United States

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Abstract

BACKGROUND: A lymphatically delivered nanoconjugate of cisplatin was evaluated in an orthotopic mouse model of locoregionally metastatic breast cancer (LABC) to determine if it can overcome some of the limitations of standard cisplatin therapy such as high systemic toxicity.

METHODS: Human breast cancer cells (10^7 MDA-MB-468LN) were injected into the mammary fat pad of female nu/nu mice. Once tumor volume reached 50 mm^3, intravenous cisplatin or subcutaneous hyaluronan-cisplatin (HA-cisplatin) nanoconjugate was given 1/week × 3 weeks at 3.3 mg/kg (platinum basis).

RESULTS: Nanoconjugates colocalized with the tumors after subcutaneous peritumoral injection and showed improved efficacy to intravenous cisplatin. After 1 month, renal tubular hemorrhage and edema were more prevalent in the intravenous formulation compared with subcutaneous HA-cisplatin nanoconjugates.

CONCLUSIONS: This nanocarrier delivery platform focuses on delivering drugs to the areas in which tumor burden is greatest, potentially reducing systemic toxicity, and has future applicability as a neoadjuvant or adjuvant therapy for LABC.

Excluding skin cancers, breast cancer is the leading cause of cancer in women today and next to lung cancer is the second most common cause of cancer deaths in women.[1] Despite the excellent short-term prognosis with current treatments, over 60% of women with localized breast cancer eventually develop distant, late-stage disease.[2]

Neoadjuvant chemotherapy is considered the standard of care for locally advanced breast cancer because it decreases tumor size allowing for subsequent breast-conservation surgery, radiation, and further adjuvant chemotherapy. The goal of neoadjuvant therapy is to not only treat locoregional and systemic disease but to also inhibit further development of micrometastases, angiogenesis, and release of serum growth factors. The major problem associated with these chemotherapeutics is toxicity, often leading to hospitalizations or other treatments. Hassett et al[3] compared outcomes within the first year of treatment among 3,526 newly diagnosed breast cancer patients 63 years or younger; 61% of chemotherapy patients were hospitalized or were treated at hospital emergency rooms compared with 42% of the patients treated without chemotherapy.

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†SC and MSC are co-first authors.

* Corresponding author. Tel.: +1-785-864-4388; fax: +1-785-864-5736.

E-mail address: mforrest@ku.edu

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Cisplatin (cis-diaminedichloroplatinum (II) [CDDP]) attacks cancer cells by promoting DNA binding and crosslinking. The most significant, dose-limiting toxicities of CDDP therapy are neurotoxicity and nephrotoxicity, both of which are strongly influenced by peak plasma concentration.[4] Additionally, most patients (as high as 75%–100%) treated with CDDP show some level of ototoxicity. This toxicity is cumulative and can be irreversible. Fractional or metronomic dosing schedules that divide the same total dose of CDDP over several smaller injections (eg, daily) have been shown to significantly reduce nephrotoxicity and ototoxicity[5,6] because of lower peak plasma concentration, but metronomic dosing requires more frequent treatments and longer in-hospital stays and leads to increased care costs, thus far limiting its use in practice. Overall toxicities associated with CDDP have led it to be considered in many cases a second-line therapy in breast cancer and typically used in combination with other cytotoxic drugs.

Currently, no therapeutic drugs are designed for locoregional lymphatic treatment, and all current chemotherapies for breast cancer are delivered systemically and have relatively poor penetration into the lymphatics. From these data, it can be concluded that there is a critical need to develop better adjuvant and neoadjuvant therapies or delivery methods that decrease local and systemic toxicity to the patient. Direct chemotherapy to the lymphatics using nanocarriers may be the solution. Localized chemotherapy avoids systemic toxicity by restricting chemotherapy agents to diseased tissue areas without subjecting other "unaffected" areas (normal tissue) to harmful drug concentrations that damage these cells irrespectively. Local chemotherapy is implemented in limb perfusions for limb-isolated melanomas and hepatic artery pumps in some hepatic cancers.[7,8] Unfortunately, no such technique exists currently for breast cancers. In this report, we describe using the unique drainage properties of the lymphatic system, along with nanoparticle drug carriers that can be targeted to the lymphatics of the breast, preventing systemic toxicity.[9]

Materials and Methods

Nanoconjugate synthesis

Hyaluronan-cisplatin (HA-Pt) nanoconjugates were formed by stirring 10% weight/volume (w/v) hyaluronan (HA, 35 kD; Lifecore Biomedical, Chaska, MN) and 4.5% w/v CDDP (Sigma Aldrich, St Louis, MO) in water, protected from light, for 4 days. The mixture was then filtered (0.2-μm nylon membrane) and dialyzed against water (10-kD cellulose tubing; Pierce, Rockford, IL) for 48 hours at 4°C and lyophilized; CDDP conjugation was determined by atomic absorption spectroscopy (SpectAA GTA-110; Varian Inc., Palo Alto, CA, USA). Nanoconjugates typically contained 25% wt/wt covalently linked CDDP.

Cell toxicity studies in vitro

The lymphatically metastatic breast cancer cell line MDA-MB-468LN was maintained in modified Eagle's medium alpha supplemented with 10% fetal bovine plasma, 1% L-glutamine, and .4 mg/mL G418.[9,10] In preceding proliferation studies, cells were trypsinized and seeded into 96-well plates (5,000 cells/well). After 24 hours, CDDP, HA-Pt (with or without silver activation), or HA was added (n = 12, 7 concentrations), and 72 hours after addition, resazurin blue in 10 μL of phosphate-buffered saline was added to each well (final concentration of 5 mmol/L). After 4 hours, well fluorescence was measured (λ_{ex} 560 nm, λ_{em} 590 nm) using a fluorophotometer (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA). IC_{50} (or inhibitory concentration, 50%) was determined as the midpoint between saline (positive) and cell-free (negative) controls for each plate. For comparison, 2 other breast cancer cell lines (MDA MB-231 and MCF-7) were tested in similar fashion with IC_{50} levels calculated.

Pathology studies

Healthy Sprague-Dawley rats (250–300 g, Charles River) were randomly divided into 2 groups and administered CDDP intravenously via the tail vein or HA-Pt subcutaneously into the mammary fat pad (1.0 mg/kg or 3.3 mg/kg platinum basis, n = 5/group). The animals were euthanized after 4 weeks, and the liver, bilateral kidneys, spleen, lungs, heart, right (ipsilateral) and left (contralateral) axillary nodes, and brain were excised intact and stored in 80% alcoholic formalin solution overnight for fixation before slide mounting. Mounting using hematoxylin and eosin staining were conducted by Veterinary Laboratory Resources (Kansas City, KS). The pathological examination was performed by a blinded board-certified veterinarian pathologist (University of Kansas Medical Center, Kansas City, KS). Animal procedures were approved by the University of Kansas Institutional Animal Care and Use Committee.

In vivo tumor model and treatment

The MDA-MB-468LN breast cancer cells were trypsinized (.25% w/v trypsin) and prepared in 1 × phosphate buffered saline solution at 3 different cell concentrations (10^5 cells/mL, 10^6 cells/mL, and 10^7 cells/mL). Cells (100 μL) were injected under pentobarbital sedation into the mammary fat pad of female nu/nu mice using a 27-G needle through a 5-mm incision (20–25 g, Charles River). The incision was closed with a sterilized staple and was removed a week after when the incision was healed. The MDA-MB-468LN cell is transformed with a green fluorescent protein so tumor growth was monitored by fluorescent whole-body imaging using a CSI Maestro imaging system (Woburn, MA), and tumor size was measured twice

a week with a digital caliper. Tumor volume was calculated by using the following equation: tumor volume (mm^3) = .52 × (width)^2 × length. Animals were euthanized before the study's end when their tumor size reached 2,000 mm^3or the body score index fell under 2. Tumors of 50 mm^3 to 100 mm^3 were observed after 3 weeks, and animals were randomly divided into 4 different treatment groups. Treatments were administered in the third and fourth weeks after tumor cells implantation.

Results

In vitro characterization of nanoconjugates

Before in vivo studies, nanoconjugates were evaluated in vitro for their ability to inhibit breast cancer cell growth. CDDP lends itself to complex formation with polycarboxylic polymers because 1 or more of the chlorides can be displaced allowing formation of a labile ester linkage with the polymer.[11] CDDP was highly conjugated to hyaluronan, with typical nanoconjugates having 25% wt/wt platinum/complex (approximately 65% conjugation efficiency) and a release half-life of 10 hours in saline. Cell toxicity was determined as the reduction in cell proliferation over 72 hours; HA-Pt nanoconjugates had similar cytotoxicity (IC_{50}) in vitro to the standard CDDP formulation in the following multiple breast cancer cell lines tested: MDA-MB-468LN, 3.9 and 3.6 μmol/L (CDDP and HA-Pt respectively); MDA-MB-231, 5.9 and 5.9 μmol/L; and MCF-7, 5.7 and 5.2 μmol/L. HA showed no toxicity at 10 mg/mL, the upper limit of testing (data not shown).

In vivo efficacy analysis in xenografts

Control animals showed a standard tumor growth curve at 10^6 cells/injection with tumor volumes exceeding 1,000 mm^3 at 6 weeks postinoculation (Fig. 1A). HA carrier-only animals showed no difference from controls, confirming in vitro data that HA has no direct anticancer activity. The intravenous standard CDDP-treated animals showed a tumor growth delay of about 3 weeks compared with controls ($P < .05$) with a median survival of 12 weeks (compared with 7 weeks in controls $P < .01$, Fig. 1B). HA-Pt–treated animals had an initial delay in tumor growth of 5 weeks ($P < .01$ compared with controls, but this was not significant compared with intravenous CDDP with both curves

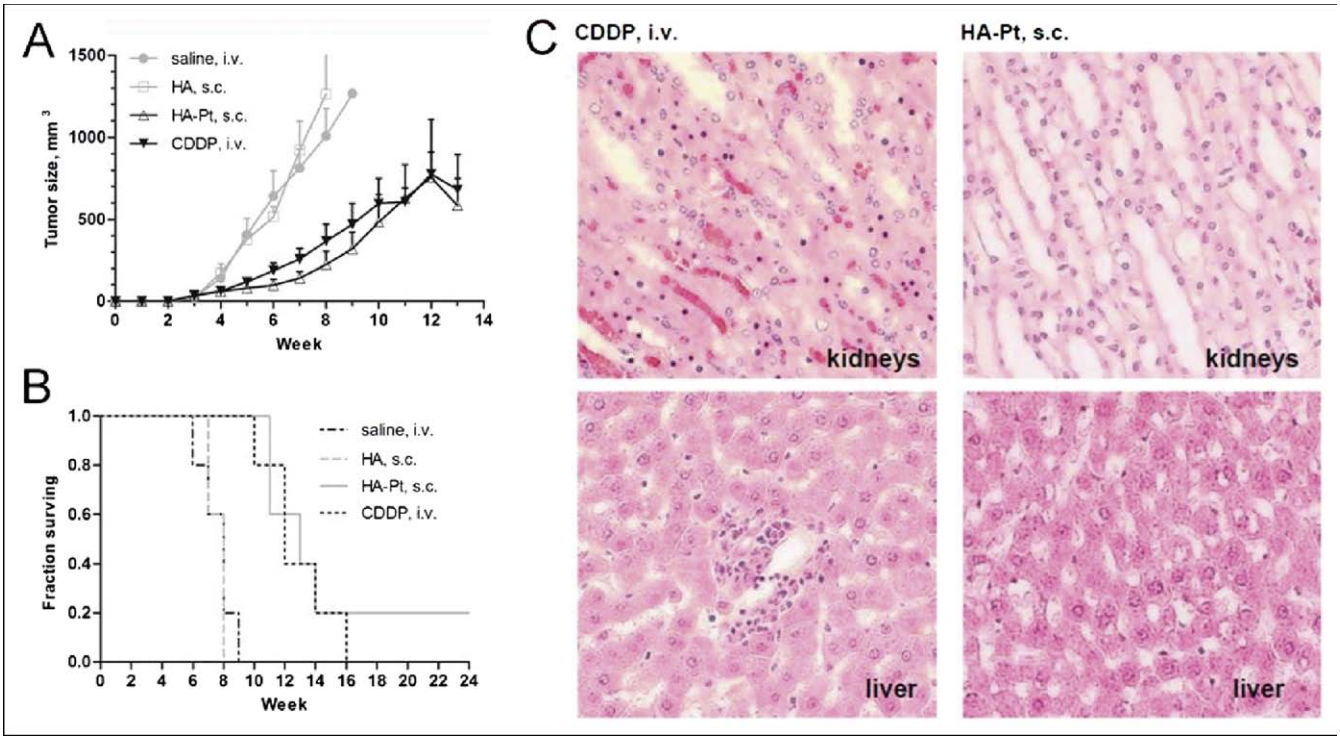

Figure 1 (A) Measurement of tumor size after orthotopic implantation of 10^6 MDA-MB-468LN breast cancer cells in nude female mice. Animals were administered saline, HA, or equivalent doses of CDDP and HA-Pt (3.3 mg/kg platinum basis). (B) Survival curves of animals treated by CDDP or HA-Pt. Survival criteria were tumor volume less than 1,000 mm^3 and no tumor ulceration or infection (n = 5). (C) Kidneys of the subcutaneous HA-Pt group had a normal appearance except for sparse minimal tubular cell necrosis, whereas the intravenous CDDP treatment group had pyknotic nuclei and apoptosis in the medullary tubular epithelia cells. Livers of the subcutaneous HA-Pt group had very minor hepatitis but otherwise appeared normal, whereas the intravenous CDDP treatment group had necrotizing lesions and hepatitis. Sprague-Dawley rats were injected subcutaneously into the mammary fat pad with HA-Pt or intravenously with CDDP (3.3 mg/kg). Slides are typical of animals in each study group (n = 5).

meeting by 12 weeks postinoculation) with a median survival of 12 weeks as well. However, there was 1 animal in the HA-Pt group who showed a true complete response to treatment with no measurable tumor and survival well exceeding 24 weeks (upper limit of study). There were no complete responders in the intravenous CDDP group.

Pathology

At the conclusion of the 30-day toxicity study, animals were euthanized, and a full pathological examination was performed. Brain tissue and underlying tissue of the injection site were noted to be normal in appearance with no microscopic changes for all study groups. Very mild changes in lymph nodes were detected for high-dose intravenous CDDP (3.3 mg/kg) and subcutaneous HA-Pt. Very mild morphological changes were observed in the livers for animals receiving both low-dose CDDP intravenously (1.0 mg/kg) and low-dose HA-Pt subcutaneously as indicated by the presence of mild inflammation in the sinusoids (Fig. 1C). Mild degeneration with some sinusoidal necroses was observed for animals receiving high-dose intravenous CDDP and high-dose subcutaneouos HA-Pt treatment. Necroses, however, were more severe in the intravenous CDDP group. In addition, 60% of animals receiving low-dose intravenous CDDP were observed to develop mild renal necrosis including hemorrhage into the renal tubules along with tubular edema. In contrast, none of the animals receiving low-dose subcutaenous HA-Pt had renal tubular necrosis. Similarly, 4 of 5 (80%) animals receiving high-dose intravenous CDDP compared with 1 of 5 (20%) animals receiving high-dose subcutaneous HA-Pt were diagnosed with mild renal tubular necrosis. Overall, the pathology studies showed that the HA-Pt conjugates had a lower incidence of both renal and hepatic toxicity compared with the conventional intravenous CDDP treatment at all dose ranges. Additionally, no neurotoxicity in the brain or local injection site toxicity in the underlying muscle tissue was observed in the treated animals (data not shown).

Comments

Locally advanced breast cancer in women remains a challenge for treatment, with current multimodality therapy resulting in moderate toxicity both locoregionally and systemically. Locoregional relapse of breast cancer can occur in up to 13% of patients, and a complete axillary lymphadenectomy can reduce this risk to less than 2% but carries its own surgical risks and morbidity including numbness in the upper medial arm, axilla and chest wall, increased incidence of skin and wound infections, and painful lymphedema in up to 30% to 50% of patients.[12–14] Cytotoxic chemotherapies also have poor penetration to the locoregional lymphatics in the breast because of separation of the lymphatics from the systemic vasculature as well as lymphatic monodirectional flow.[15] Platinum-based chemotherapy is the most commonly used chemotherapeutic in the United States but carries its own morbidity including dose-limiting nephrotoxicity and neurotoxicity. CDDP is not commonly used as a single-agent treatment for breast cancer although it is a part of several combination regimens, but CDDP may have a place in patient populations that have failed to respond to anthracyclines and taxanes. Triple-negative breast cancers are commonly resistant to standard regimens, but there is increasing evidence that these patients may have increased platinum sensitivity.[16] Recent studies report that BRCA1 breast cancers are highly sensitive to platinums because of the role BRCA1 plays in DNA double-strand repair.[17,18]

Lymphatically delivered chemotherapy through a subcutaneous injection is a novel approach to drug delivery that has only recently been shown by our group to be feasible with CDDP.[9] Nanoconjugation of CDDP to hyaluronic acid not only allows for improved locoregional delivery of the drug to the site of the greatest tumor burden in the breast and axillary tissues but also decreases the level of renal toxicity associated with this drug. Our toxicity data show that there is no significant injection site toxicity on pathological analysis, indicating that when bound to the carrier, CDDP does not lead to necrosis of the surrounding tissue. It is only after the carrier is cleaved from the conjugate by either hyaluronidase or receptor-mediated endocytosis (hyaluronan is a ligand for CD44 receptors overexpressed on lymph nodes and many cancers including breast cancers and melanoma) that the drug becomes functionally active. In our formal tissue toxicology analysis, both renal and hepatic toxicity was significantly reduced in the HA-Pt–treated animals compared with the standard CDDP-treated group.

The other benefit noted in the nanoconjugate group involved efficacy in tumor growth inhibition and response in vivo. The HA-Pt versus CDDP tumor growth curves in Fig. 1A show that both drugs effectively delay tumor growth in an orthotopic, lymph node metastatic model of breast cancer. The HA-Pt group had an improved although not statistically significant arrest in tumor growth (about 2–3 weeks of additional delay compared with CDDP and a 5- to 6-week delay compared with controls). What is significant is that a complete response was seen in 20% of the HA-Pt–treated group and in 0% of the CDDP-treated group (Fig. 1A and B). These data support that HA-Pt injected subcutaneously in the breast has mildly improved efficacy over standard CDDP injected intravenously. With improved efficacy and reduced toxicity with the nanoconjugate formulation in a metastatic breast cancer model in vivo, these data provide solid support for completing further preclinical proof of concept studies to advance this formulation into clinical applications. The benefits of a locally injectable chemotherapeutic over an intravenous infusion include potentially lower cost because the patient does not have to be attached to an infusion pump with nursing or physician supervision as well as the ability to deliver CDDP weekly with the sustained release properties of the nanoconjugate as

opposed to daily in most current intravenous protocols. The sustained-release properties of this nanoconjugate provide an excellent boost to locoregional tumor tissues while maintaining therapeutic systemic levels and provide promise for future potential use in locally advanced breast cancer in both the neoadjuvant and adjuvant setting. In addition, localized therapy may be an effective addition to systemic therapy in patients with metastatic disease; localized therapy can provide a higher dose of chemotherapeutic in the most at-risk tissues than is possible with systemic therapy alone. Other published and ongoing studies have shown that HA-Pt given locoregionally provides adequate systemic levels of CDDP including serum area under the curve levels greater than intravenous CDDP but without the high (toxic) peak serum concentrations of intravenous therapy.[9] As a result, we hypothesize that these nanoconjugates would have a useful role in the treatment of locally advanced breast cancer in the neoadjuvant setting, providing enhanced locoregional drug efficacy while maintaining or even enhancing systemic therapy to distant disease. Further studies will be necessary to evaluate long-term efficacy and toxicity in animal models as well as the role of this nanoconjugate in combination chemotherapy regimens.

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Discussion

Kelly McMasters, M.D. (Louisville, KY): President Allo, Dr. Scaife, members, and guests, first I would like to take a moment to thank you for inviting me as President of the Southeastern Surgical Congress this year to the meeting. I think it is a nice tradition that the President of the Southeastern gets invited to the Southwestern and vice versa, and I am happy to be here.

Dr. Cohen, congratulations on a very nice study. The authors describe a nanodelivery system for intralymphatic delivery using hyaluronan. I have a few questions. First, why cisplatin? Cisplatin is not a drug commonly used for breast cancer, at least not for first-line treatment. Presumably, you can conjugate other chemotherapeutic drugs to this nanocarrier, and why did you choose cisplatin? Second, you have answered this in your presentation, but I had some questions regarding the article that you might want to clarify. You actually do get higher doses of chemotherapy into the lymph nodes, and there is evidence that there is intralymphatic transport, but, in terms of treatment of the primary breast tumor, is the mechanism intralymphatic or is it a local or is it a systemic effect? You state in the article that the cisplatin is only released by either hyaluronidase or receptor-mediated endocytosis in tumor cells. Do you have any evidence that receptor-mediated endocytosis specifically in breast cancer cells is taking place? Finally, how do you think this should be used clinically? For locally advanced breast cancers, we use neoadjuvant chemotherapy with 80% or 85% response rates. It is hard to think that we are going to improve upon that very much with this type of treatment, but where does it make sense to use this? We use systemic chemotherapy presumably also to prevent systemic recurrences. Does this type of treatment also have a systemic effect?

Mark S. Cohen, M.D., F.A.C.S. (Kansas City, KS): Why did we pick cisplatin specifically? You are correct; it is not a typical first-line agent for breast cancer treatment. We chose cisplatin as a model because platinum as a heavy metal is a very easy agent to accurately quantify in tissues and serum. When using atomic absorption spectroscopy,

one can determine exactly how much platinum is present in any given piece of tissue. We have looked at this with other drugs that are more applicable to breast cancer, and those are contained in separate manuscripts in press. With regard to your second question, as far as the primary breast tumor itself, when we deliver this nanoconjugate, it is done as a subcutaneous injection locally at the tumor site, so you do get some local uptake into the primary tumor. In terms of how the agent is taken up in the cells, even in culture when you do not have the enzyme hyaluronidase present, the carrier still gets taken up intracellularly. It has been shown that hyaluronan is a ligand for CD44 receptor on the cell surface, which is overexpressed in cancer cells such as breast and melanoma. It is believed that the carrier and therefore nanoconjugate may be directly taken up into the cell at least partially through receptor-mediated endocytosis, and, in our in vitro experiments, we do see transference of fluorescence intracellularly when the particles are fluorescently tagged. With regard to your last question, we do see adequate systemic levels when we give a subcutaneous injection of our nanoconjugate, and, if you measure area under the curve in the serum, we actually can achieve a slightly higher level than we see with an intravenous dose of cisplatin. The benefit of our drug carrier system is that you get a nice sustained release of drug off the carrier without the high c-max peak levels seen by first pass kinetics. We believe this might protect organs from exposure to high peak concentrations of drug that result in toxicity, thus allowing one to potentially deliver higher doses of drug with less toxicity. It is this ability of our delivery system to maintain therapeutic systemic levels that supports its use for the treatment of patients with metastatic disease outside of the local regional basin.

Courtney Scaife, M.D. (Salt Lake City, UT): There is some evidence in colon cancer and ovarian cancer using other nanoparticle models that there is evidence of decreased metastases. Have you used a metastatic model?

Mark S. Cohen, M.D., F.A.C.S. (Kansas City, KS): This model of breast cancer metastasizes both to the lymph nodes and then to the lung. We have observed decreases in metastatic disease and improved survival in this model with prolonged treatment. Those data have been submitted as a separate manuscript, so in answer to your question, yes we do see a nice effect on systemic disease with this nanoconjugate.

Barb Pockaj, M.D. (Phoenix, AZ): Even though the cisplatinum is not first line for patients with breast cancer, there is some suggestion that carboplatinum and Taxol are actually first line in triple negative disease and there are studies ongoing with regard to that chemotherapy combination. Have you looked at this and different subtypes of breast cancer?

Mark S. Cohen, M.D., F.A.C.S. (Kansas City, KS): One of the cell lines I presented in our in vitro data was the MDA-MD-231, a triple negative cell line. Even here, we see equivalently good cell death in these cells on our dose-response curves. For a clinical application in the future, we would envision it in patients with hard to treat tumors such as local advanced breast cancer patients who are triple negative; this nanoconjugate therapy could be delivered weekly or even biweekly and combined with other agents such as Taxol.