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Subcutaneous delivery of nanoconjugated doxorubicin and cisplatin for locally advanced breast cancer demonstrates improved efficacy and decreased toxicity at lower doses than standard systemic combination therapy in vivo

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Abstract

BACKGROUND: Combination cytotoxic agents in breast cancer carry dose-limiting toxicities. The aim of this study was to test the hypothesis that nanocarrier-conjugated doxorubicin and cisplatin would have improved tumor efficacy with decreased systemic toxicity over standard drugs, even at lower doses.

METHODS: Female Nu/Nu mice were injected in the breast with human MDA-MB-468LN cells and treated with either standard or nanocarrier-conjugated combination therapy (doxorubicin plus cisplatin) at 50% or 75% maximum tolerated dose (MTD) and monitored for efficacy and toxicity over 12 weeks.

RESULTS: Efficacy results for mice treated with hyaluronan-conjugated doxorubicin and cisplatin at 50% MTD were as follows: 5 complete responses, 2 partial responses, and 1 case of stable disease. For hyaluronan-conjugated doxorubicin and cisplatin at 75% MTD, efficacy results were as follows: 7 complete responses, 1 partial response. All complete responses were confirmed histologically. In comparison, mice given standard doxorubicin and cisplatin at 50% MTD demonstrated progressive disease in 6, stable disease in 1, and partial response in 1. For standard doxorubicin and cisplatin at 75% MTD, there were 5 cases of progressive disease and 3 of stable disease ($P < .0001$ on multivariate analysis of variance). At 75% MTD, standard drug–treated mice had significant weight loss compared to nanocarrier drug–treated mice ($P < .001$).

CONCLUSIONS: Subcutaneous nanocarrier delivery of doxorubicin and cisplatin demonstrated significantly improved efficacy with decreased toxicity compared with standard agent combination therapy at all doses tested, achieving complete pathologic tumor response.

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Breast cancer accounted for >209,000 new cases of cancer in 2010, with >42,000 deaths in the United States alone, making it the leading cause of cancer in women (excluding skin cancers) and the 2nd leading cause of cancer death in women after lung cancer.[1] Although current treatments often carry excellent short-term prognoses, up to 13% of women will develop locoregional recurrence within 9 years of initial treatment, and up to 25% of these women will have distant metastatic disease at the time of recurrence.[2–4] Also, >60% of women with localized breast cancer will eventually develop distant, late-stage disease.[5]

For women with locally advanced breast cancer, standard-of-care treatment includes neoadjuvant chemotherapy followed by surgical resection, radiation, and further adjuvant chemotherapy. One goal of the neoadjuvant chemotherapy is to decrease locoregional tumor burden and tumor size to decrease surgical morbidity, allowing in many cases breast conservation. Additionally, neoadjuvant treatment can inhibit further advancement of disease, including the development of further metastatic spread. However, the utility of combination cytotoxic chemotherapy is often limited by systemic toxicities, which can be severe or dose limiting in many cases. One recent study demonstrated that 61% of women diagnosed with breast cancer who received chemotherapy were hospitalized for complications, compared with only 42% of patients not receiving chemotherapy.[6]

Several classes of chemotherapeutic agents are used in both neoadjuvant and adjuvant treatment regimens for breast cancer. A common 1st-line agent is doxorubicin, which is from a class of drugs called anthracyclines. Doxorubicin decreases cancer growth by inhibiting deoxyribonucleic acid intercalation and macromolecular biosynthesis within cancer cells. Significant toxicities associated with doxorubicin include neutropenia, alopecia, and cardiac toxicities such as congestive heart failure and dilated cardiomyopathy. Cardiomyopathy from doxorubicin is a toxicity related to the cumulative dose of the drug and occurs in up to 4% of patients, often as a late finding even up to 1 year after the completion of treatment.[7] Another class of chemotherapeutic agents commonly used in combination therapy for breast cancer is platinum agents, such as cisplatin or carboplatin. Cisplatin inhibits cancer growth by promoting deoxyribonucleic acid binding and cross-linking, thereby triggering apoptosis. This drug also carries systemic toxicities, the most notable being neurotoxicity, ototoxicity, and nephrotoxicity, which have been demonstrated to be related to high peak plasma concentration levels.[8] In fact, >75% of patients receiving cisplatin develop some level of ototoxicity, which is cumulative and can be irreversible.[9]

Although these agents can be reasonably effective in the adjuvant setting, their moderate toxicity profiles create a critical need to improve the safety and tolerability of combination regimens as well as enhance their efficacy even further. The use of nanoconjugation with current chemotherapeutic agents provides a novel method for drug delivery through the locoregional lymphatics, creating improved delivery of drug and cancer targeting with lower systemic toxicity while maintaining therapeutic systemic levels.[10] We have demonstrated that the nanoscopic-sized molecular weight of hyaluronan (HA) can be combined with a chemotherapeutic, allowing the drug to be preferentially taken up initially by locoregional tissues and lymphatic channels without systemic bolus release, because of the size and hydrophilicity of the conjugate.[10] Also, the nature of this construct would allow for sustained-release kinetics, allowing for improved efficacy at decreased doses.[11] We have reported in vitro and in vivo models of nanoconjugated HA-doxorubicin and HA-cisplatin in breast cancer models. These studies have shown improved delivery of the chemotherapeutic agent to the lymphatic system with a decreased toxicity profile compared with the standard agent at all doses tested, including lower drug doses.[12,13]

We hypothesized that combination therapy with doxorubicin and cisplatin when conjugated to nanoscopic HA as a drug-delivery carrier to the locoregional tissues and lymphatics would have improved efficacy at significantly lower dose, with better lymphatic penetration and a markedly reduced toxic profile, than standard combination therapy with these drugs. The aim of this study was to examine and compare with standard drugs the efficacy and toxicity of this combination HA-doxorubicin and HA-cisplatin therapy in vivo using an orthotopic murine model of a locally advanced breast cancer.

Methods

Cell culture

The lymphatically active metastatic breast cancer cell line MDA-MB-468LN (obtained as a gift from Dr Chambers and coworkers[14]) was maintained in modified Eagle's medium α (Sigma-Aldrich, St Louis, MO), supplemented with 10% fetal bovine serum, 1% L-glutamine, and .4 mg/mL G418 (Sigma-Aldrich). Adherent monolayer cultures were maintained in T-75 culture flasks and incubated at 37°C with 5% CO_2 until they achieved 85% confluency. The cells were trypsinized using .25% trypsin (Sigma-Aldrich) and passaged into T-75 flasks at a density of 1×10^6 cells. On experiment days, cells were trypsinized and counted via hemocytometer to determine the number of viable cells.

In vivo tumor model and treatment

All animal studies were done in accordance with the University of Kansas Institutional Animal Care and Use Committee guidelines. Lymphatic breast tumor metastasis was induced in nude mice according to the procedure of Chambers et al,[14] who were kind enough to provide the lymphatically metastatic breast tumor cell line MDA-MB-

468LN. MDA-MB-468LN breast cancer cells were prepared in a 1× phosphate-buffered saline (PBS) solution at a concentration of 1×10^6 cells/100 μL. Cells (100 μL) were injected under isoflurane anesthesia into the right 1st breast mound (abdominal mammary fat pad) of female Nu/Nu mice aged 4 to 6 weeks using a 25G needle (20–25 g; Charles River Laboratories, Wilmington, MA). Tumor size was measured 3 times weekly using a digital caliper and confirmed by 2 separate observers. Tumor volume was calculated using the following equation: tumor volume (mm^3) = (π/6) × (width)^2 × length. When tumors reached a minimum volume of 30 mm^3, mice were randomized into control (PBS or HA) or 1 of 4 combination treatment groups (50% maximum tolerated dose [MTD] doxorubicin plus 50% MTD cisplatin [dox-cis 50], 75% MTD doxorubicin plus 75% MTD cisplatin [dox-cis 75], 50% MTD HA-doxorubicin plus 50% MTD HA-cisplatin [HA-dox-cis 50], and 75% MTD HA-doxorubicin plus 75% MTD HA-cisplatin [HA-dox-cis 75]). Pharmaceutical-grade doxorubicin and cisplatin were used for the standard treatment groups as well as to create the nanocarrier formulation, as previously described.[13] The HA control and HA treatment groups were administered subcutaneously 1 to 3 mm away from the site of tumor implantation, and the PBS control and standard treatment groups were administered intraperitoneally. The MTD level reported in mice for doxorubicin is 8 to 10 mg/kg/wk intraperitoneal dose and for cisplatin is approximately 10 mg/kg/wk intraperitoneal dose.[15,16] All treatments were given once a week for a total of 3 weeks, and mice were monitored for an additional 9 weeks upon the completion of treatment (total study period of 12 weeks). Mice were euthanized before completion of the experiment if the tumor reached >20 mm in diameter, if weight loss was significant, or if body score markedly deteriorated.

Pathologic studies

Two Nu/Nu mice from each of the treatment groups were euthanized 1 week after the completion of treatment (week 4), and an additional 2 mice from each group were euthanized at the completion of the study for histologic analysis of tumor, organ, and injection sites. The tumor site with surrounding skin, heart, lungs, brain, bilateral kidneys, spleen, liver, bone marrow from spine and femur, and ipsilateral (right) as well as contralateral (left) axillary lymph nodes were harvested intact from the mice and stored in 10% formalin solution for fixation overnight before slide mounting. Mounting using hematoxylin and eosin staining was conducted by the University of Kansas Medical Center Department of Pathology, and histologic examination was performed by a blinded, board-certified pathologist. Slide images were obtained using Aperio version 10.0 software (Aperio Technologies, Inc, Vista, CA).

Statistical analysis

Comparisons of differences between ≥2 means were determined using Student's unpaired *t* test (2 means) and Fisher's exact test. Multivariate analysis was performed using 2-way analysis of variance followed by Duncan's multiple range test (≥2 means) and Bonferroni's post hoc testing using SPSS version 17.0 (SPSS, Inc., Chicago, IL). Significance was defined at $P < .05$.

Results

In vivo efficacy analysis

To examine the efficacy of HA-doxorubicin and HA-cisplatin in vivo, tumor volumes were monitored in the mice and confirmed by histologic analysis. The control animals (PBS and HA only) demonstrated a standard tumor growth curve with tumor volumes exceeding 1,200 mm^3 by 6 weeks after inoculation (Fig. 1). There was no difference noted in tumor growth curves between PBS controls and HA (carrier only) control animals, confirming that HA by itself has no direct antitumor activity. These groups were combined as a composite control curve (Fig. 1). Of the experimental

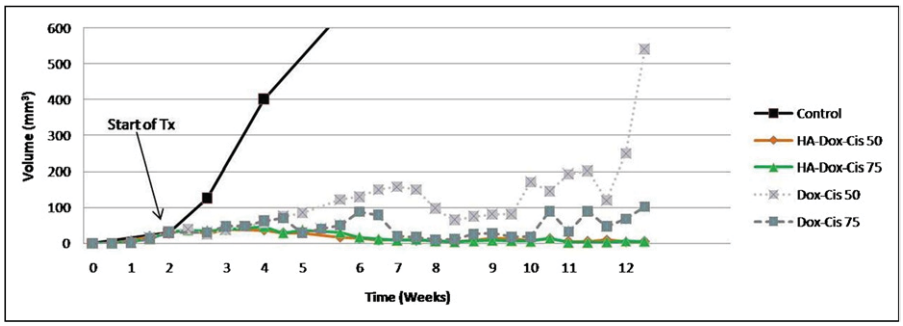

Figure 1 Comparison of breast tumor volumes with treatment. Graph shows a composite curve of the animals in control and 4 treatment groups (HA-dox-cis 50, HA-dox-cis 75, dox-cis 50, and dox-cis 75; n = 8 for each group). The control curve is a composite curve of HA carrier subcutaneous injection and 1× PBS systemic injection (n = 4 for each). Note that there is a delay in tumor growth with standard doxorubicin and cisplatin treatment, but progressive disease does still occur, whereas there were significantly more complete responders in the HA treatment groups, which was durable.

groups, HA-dox-cis 75 was noted to have the best overall efficacy, with 100% of the mice showing response to treatment and 7 of 8 mice (87.5%) having complete responses and the remaining mouse having a partial response with an 87% reduction in tumor volume (Fig. 1). The second best group for efficacy was the HA-dox-cis 50 group, in which 7 of 8 mice (87.5%) had significant responses to treatment (5 complete responses and 2 partial responses), with the remaining mouse having stable disease (Fig. 1). Alternatively, in the standard treatment groups at comparison MTD levels, the dox-cis 75 group had only 2 of 8 animals (25%) with partial responses to treatment, with the remaining 6 animals having either stable disease (n = 3) or progressive disease (n = 3). Finally, in the dox-cis 50 group, there was only 1 partial response (12.5% response rate), 1 animal with stable disease, with the remaining 6 animals (75%) with progressive disease (Fig. 1). Of note, there were no complete responders noted in either of the standard treatment groups, and in the HA-dox-cis 75 mice, all complete responses were true pathologic complete responses. An overall comparison of all 4 treatment groups using a multivariate analysis was noted to be statistically significant at $P < .0001$, and when breaking this down to compare individual groups, the response rate among the HA-dox-cis 50 group compared to the dox-cis 50 group and the dox-cis 75 group was noted to be statistically significant ($P = .0004$ and $P = .005$, respectively). Conversely, comparing the HA-dox-cis 75 group to the dox-cis 50 and dox-cis 75 groups was also noted to be statistically significant ($P < .0001$ and $P = .0003$, respectively). Of note, comparison between the 2 dose levels of the standard treatment was not noted to be statistically significant ($P = .27$).

Pathologic analysis

In the complete responders of both the HA-dox-cis 50 and the HA-dox-cis 75 treatment groups, no visible tumor could be seen grossly (Fig. 2A) compared with the visible tumors in the standard dox-cis 50 and dox-cis 75 groups (Fig. 2B). To confirm the significance of these findings, the tumor sites, as well as bilateral axillary lymph nodes, heart muscle, and kidneys, were examined histologically for all treatment groups. The tumor site and lymph nodes were examined for evidence of residual microscopic cancer disease, and the heart and kidneys were examined for evidence of systemic toxicity. On histologic examination, both HA treatment dosing groups showed fibrosis and neutrophil infiltration but no histologic evidence of residual tumor at the tumor site (Fig. 2C) compared with the standard treatment at both doses, which had residual tumor with associated central necrosis (Fig. 2D). Additionally, there was no evidence of lymph node metastases present in any of the

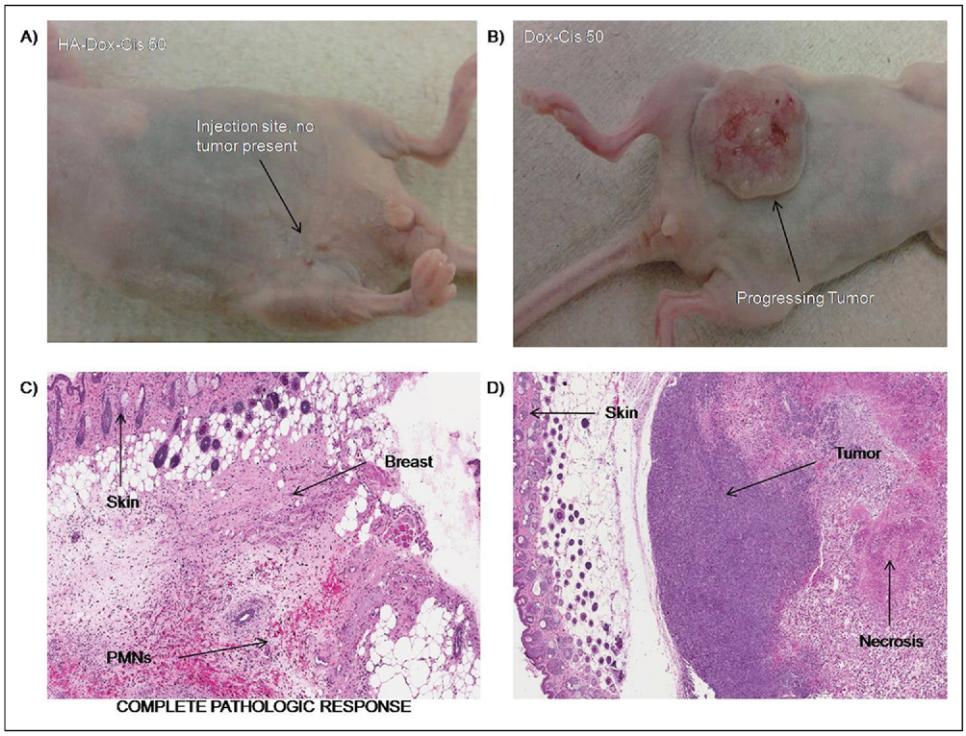

Figure 2 Evaluation of efficacy by histologic confirmation. (A) Whole-body image of mouse treated with HA-dox-cis 50 at week 12. Arrow denotes no clinical evidence of residual tumor and normal-appearing skin at the injection site. (B) Whole-body image of mouse treated with dox-cis 50 at week 12. Here, the arrow notes progressive tumor growth with ulceration after treatment. (C) Hematoxylin and eosin–stained histologic image at 7.8× magnification of mouse from (A). Arrows denote skin, normal breast tissue surrounding injection site with polymorphonuclear leukocyte infiltration, and associated fibrosis. Of note, there is no histologic evidence of tumor present, indicating a complete pathologic response. (D) Hematoxylin and eosin–stained histologic image at 5.4× magnification of mouse treated with dox-cis 50 demonstrating a partial response clinically. Arrows denote skin and histologic presence of tumor with associated central necrosis.

treated animals, while >80% of controls developed metastatic disease to lymph nodes and lungs. Finally, evaluating organ and bone marrow toxicity, there was no evidence with the short-term dosing used in this study of any histologic toxicity at the injection site, bone marrow, heart, or kidneys in any of the treated groups. However, systemic disease was noted histologically as spinal metastases in one of the mice at the 50% MTD systemic doxorubicin and cisplatin combination, whereas none of the mice in the HA groups demonstrated any systemic disease.

In vivo toxicity analysis

In addition to histologic toxicity, all mice were evaluated for signs of weight loss or deterioration in body conditioning score as a clinical sign of toxicity. All the animals in both HA groups had no sustained weight loss or deterioration in body score throughout the study. Also, there was no significant difference noted between either HA dosing group with respect to weight loss ($P = .4917$). In comparing the weight-loss profiles of the HA groups with dose-matched standard drug combinations, it was noted that there was no weight loss noted in the standard 50% group as well, but there was an average weight loss of 23% from baseline in the animals from the dox-cis 75 group, which was noted to be statistically significant ($P < .001$; Fig. 3A). It should be noted, though, that the HA-dox-cis 75 group did demonstrate some weight loss (average, 10%) while receiving the 3 weeks of treatment, but this effect was transient, with all mice returning to their baseline weights within 10 days after completion of treatment. This effect was permanent in the standard groups, with deterioration in body score requiring early euthanasia because of this toxicity, particularly in the dox-cis 75 group, in which 5 animals were sacrificed for clinical toxicity before completion of the study (Fig. 3B).

Comments

Locally advanced breast cancer remains a challenge to treat successfully. Available chemotherapeutic agents, although moderately effective, can result in significant local and systemic toxicities. Surgical intervention in the form of complete breast resection and axillary lymphadenectomy carries its own morbidity, including risks of nerve injury, skin and wound infections, and painful lymphedema, which has been reported to occur in >30% of patients who also receive radiation and in 10% to 20% of patients receiving lymphadenectomy alone.[17,18] Another important therapeutic challenge is that when cytotoxic chemotherapies are given systemically, they have poor penetration into the breast tissue and lymphatic system, in part because of the unidirectionality of lymphatic flow and the separation of the lymphatics from the systemic vasculature.[19] As a result, only a small dose of the drug finally reaches the tumor tissue or lymph nodes draining the tumor site.

Lymphatically delivered chemotherapy is a novel drug delivery approach that has been shown to be effective in breast cancer using single agents such as cisplatin or doxorubicin in conjugation with a nanoscopic molecular weight of HA. We have reported that in vivo use of this carrier with cisplatin or doxorubicin demonstrated improved locoregional delivery of the drug to the site of greatest tumor burden in the breast and axillary tissues with improved efficacy and decreased toxicity compared with the standard drug formulations.[12,13] In practice, however, chemotherapy for locally advanced breast cancer is multidrug, often involving a platinum agent, a taxane, and/or an anthracycline in combination. One of the pitfalls of combination systemic therapy is the added toxicity of 2 or 3 drugs over a single agent, so we chose in this study to evaluate not only the response of the combination of drugs when conjugated to

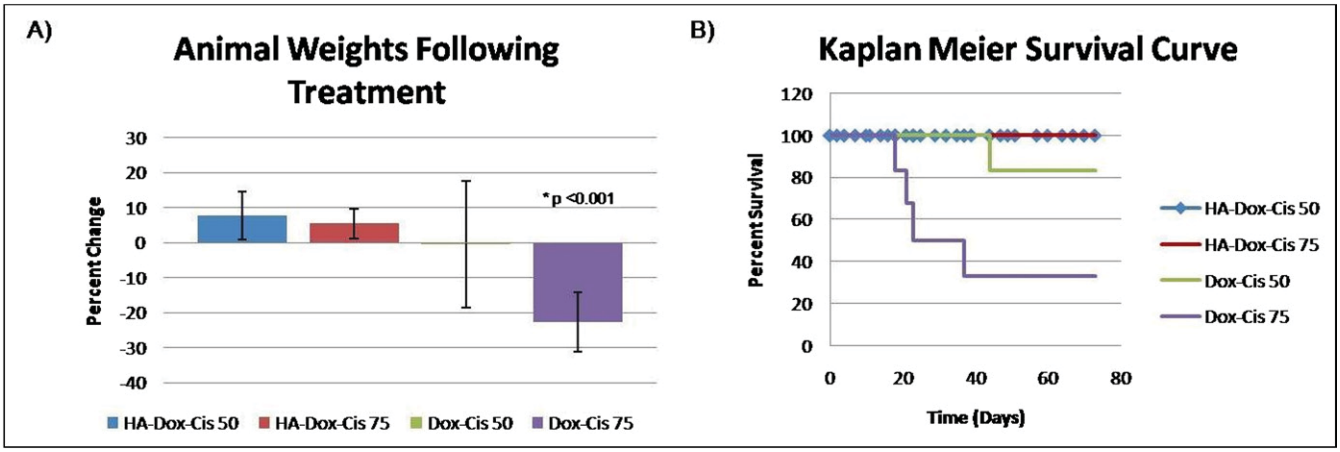

Figure 3 (A) Clinical evaluation of animal toxicity by weight changes. Of note, there was a 23% weight loss observed in the dox-cis 75 group compared with no durable weight loss in the HA-dox-cis groups, which was statistically significant ($P < .001$). (B) Kaplan-Meier survival curves by group. Note that both HA-dox-cis groups had 100% survival throughout the study, which was superior to the doxorubicin and cisplatin groups. There were 6 animals in each group, as 2 animals were euthanized immediately after treatment for histology. Note that controls were all euthanized by week 7 because of advanced tumor volumes and deteriorating body condition from progressive disease per established animal protocol end points and therefore are not included in the figure.

the nanocarrier but also the toxicity profiles of the combination when given subcutaneously. Our data demonstrated that the HA combination generated less locoregional and systemic toxicity than standard systemic agents at similar dose levels and that a reduced dose of each drug could be administered to achieve similar efficacy. This would have significant advantage clinically if lower doses can be administered in an effort to avoid dose-limiting toxicities of these agents. We observed that lower doses of each drug in combination given via the nanoconjugate peritumoral route achieved the same efficacy as higher doses required when given systemically, suggesting a possible synergistic effect in combination when combined to the nanocarrier. With respect to timing and delivery, each drug is injected individually in the subcutaneous peritumoral area, one immediately after the other. If there is extensive regional lymph node involvement, which could obstruct the lymphatics, it would be possible to inject the drug just proximal as well as distal to the tumor mass to ensure adequate uptake in the entire lymphatic basin. In terms of the mechanism of this systemic effect, once the HA is cleaved in the lymphatics or peritumorally by the enzyme hyaluronidase, which is present in lymph, the free drug can either interact locally at the tumor cell by diffusion or active transport into the cell or will be transported because of its smaller size into the systemic circulation, where it will achieve therapeutic systemic levels. The difference between this delivery and intravenous infusion therapy is that the cleavage rate of free drug off the carrier provides a slow, sustained release of drug with a lower C_{max} but achieves equivalent plasma areas under the curve over time, allowing the drug to be effectively therapeutic to systemic metastases as well. Systemic absorption was measured in the nanoconjugates individually in previous studies of these compounds and compared with standard agents. Those studies demonstrated comparable levels of systemic penetration via equivalent plasma areas under the curve.[12,13] Intratumoral as well as lymphatic levels of HA-cisplatin compared with systemic cisplatin were also measured, demonstrating significantly increased levels of cisplatin in the tumor and lymphatic tissues in the HA-cisplatin group compared with systemically delivered cisplatin.[13]

Although previous studies have demonstrated improved efficacy and pharmokinetic profiles of nanoconjugated chemotherapeutics as single agents in vivo, the use of combination therapy more closely approximates treatment of breast cancer clinically. Systemic chemotherapeutic agents are often administered in combination because of synergistic effects. Therefore, the combination of 2 nanoconjugated agents in vivo would be expected to further enhance this synergy. Although individual uptake of each drug was not measured intratumorally in the combination therapy, on the basis of the dramatically improved efficacy of the combination nanoconjugated agents compared with systemic agents in combination as well as the previously published single-agent data, it stands to reason that uptake of these agents is improved. Also, because of the reduced toxicity profile of this delivery system, both nanoconjugated agents can be delivered simultaneously, allowing for increased tumor targeting. In the study, half of the animals in both the 50% and 75% HA-dox-cis treatment arms were given both injections at the same site peritumorally, while the other half of the animals received each injection on opposite sides of the tumor. No difference in tumor response was noted between the difference in injection sites.

The results in this study demonstrated that in combination, HA-dox-cis was able to generate a complete pathologic response in a majority of animals treated even at only 50% of the MTD levels of the standard doxorubicin and cisplatin combination. When this dose was increased to 75% MTD, the HA-dox-cis group developed a complete pathologic response in 87.5% of animals treated, with the remaining animal having a partial response with 87% tumor reduction. Comparatively, neither of the standard dosing groups had any complete responders, indicating significantly improved efficacy for the nanocarrier-delivered drug combination even at half of the standard dose of current therapy.

With regard to toxicity, the standard treatment at 75% MTD of doxorubicin and cisplatin resulted in significant morbidity and mortality, with 67% of the mice requiring euthanasia before study completion because of significant clinical toxicity, as evidenced by decreased body scores and long-term weight loss. Alternatively, this was not seen in either HA group, although a transient 10% weight loss was noted in the 75% MTD HA group during the treatments, which resolved spontaneously. From a histologic standpoint, no evidence of cardiac or renal toxicity was noted in any of the groups, although cardiac toxicity is due to a cumulative dose of doxorubicin, and this cumulative effect was not likely achieved with only 3 doses of drug given. Furthermore, differences in renal toxicity may not have been observed in this small group either when only 3 doses of drug are given, all at $\leq$75% of their maximum clinical dose. Further investigation with longer follow-up and longer dosing regimens will provide more insight regarding chronic toxicity of the HA combination treatment.

Overall, we conclude that on the basis of this study, nanoconjugated combination therapy with doxorubicin and cisplatin exhibited potent anticancer activity against a locally advanced breast cancer orthotopic murine model in vivo. These data indicate that this combination therapy has improved efficacy (especially locoregionally) with decreased clinical toxicity compared with standard dosing of doxorubicin and cisplatin combinational therapy. The limitations of this study include a small sample size for each group as well as a short (3-week) duration of therapy. Despite these limitations, there was enough of an improvement in efficacy and toxicity with HA-dox-cis at all dosing levels over standard therapy to demonstrate statistical significance.

As this system uniquely targets and boosts drug delivery to the primary tumor, lymphatics, and locoregional tumor

bearing tissues, it is uniquely suited for patients who have extensive regional nodal disease. Clinically, this novel delivery platform would need to be evaluated 1 drug at a time as per US Food and Drug Administration regulations for safety and efficacy in patients before any combinational therapy. In this regard, we would plan to first test each nanoconjugate given peritumorally subcutaneously as an additive to standard-of-care neoadjuvant systemic therapy in a locally advanced breast cancer population. Although the nanoconjugated drug should provide a locoregional boost to therapy, which could improve regional control and treatment, doxorubicin systemic levels, as we have shown in rodents, will achieve an area under the curve in the plasma equivalent to that generated by systemic agents and therefore should also be therapeutic systemically. One benefit of using the nanoconjugate is that its sustained release kinetics provide for a lower (less toxic) C_{max} level in the plasma. We would expect that HA-doxorubicin, therefore, would have excellent efficacy on systemic metastatic disease, which these patients undoubtedly harbor. Clinical use of this nanodelivery for doxorubicin would provide the opportunity to evaluate the added benefit of the locoregional boost on the primary tumor and lymph nodes at the time of surgery and axillary lymphadenectomy as well as the effect on any known systemic metastatic disease and be able to compare this effect to standard systemic therapy alone. Treatment with the nanoconjugate should reduce the tumor burden and lymphatic disease before surgical resection in hopes of preventing future recurrence or in patients who have locoregional recurrence and have failed traditional systemic agents or are limited in the administration of these agents because of cumulative dose toxicity. In patients with known concomitant systemic disease, as these agents have systemic penetration, they could be effective at targeting the systemic disease or could provide a useful adjunct to traditional systemic therapy, allowing a reduced dose of the systemic agent. These data provide a solid foundation for further translation of this delivery system toward a wide range of clinical applications where there may be need for novel treatment strategies that carry less toxicity and morbidity to patients.

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Discussion

Dr Maria Allo (San Jose, CA): Thank you Dr Cohen for a very nice presentation. This is a very exciting area in oncology right now. Besides the work that your group has done in breast cancer, there has been a considerable amount of work done in other centers in prostate cancer models and also using different kinds of nanoconjugates. I have several questions for you.

First, is the efficacy of this treatment affected by the presence or absence of CD44 receptors in the tumor being treated?

The second question is, Is the systemic level of the nanoconjugated drug influenced by the degree to which the

drug is taken up in the tumor or does the amount not taken up get eliminated in its nanoconjugated form and therefore not affect systemic uptake?

The third question actually has to do with some of your previous work that was reported. I wonder why you didn't have an arm that looked at HA cis versus HA cis-doxy. How, if at all, did the addition of the doxorubicin influence your outcomes? Also, what, if any, were challenges of adding the doxorubicin to your nanoconjugated hyaluronan and cisplatin?

Thank you for a nice presentation and for providing the manuscript in advance.

Dr Cohen (Kansas City, KS): Thank you Dr Allo. To address the first question, hyaluronic acid is a ligand for the CD44 receptor which is overexpressed in many breast cancers. This aids in the delivery of the nanoconjugated breast cancer drug to the tumor sites; however, hyaluronidase is present in lymphatic tissues as well as in the lymphatic fluid and hyaluronidase is able to cleave the hyaluronic acid carrier from the drug to provide a direct delivery of the drug locoregionally to the tumors so it is able to penetrate tumor-bearing tissue even without overexpression of CD44. The CD44 overexpression in these tissues just enhances that delivery effect at the tumor site. With regard to your second question, we have completed pharmacokinetic curves looking at the systemic response of these drugs, specifically at serum drug levels and the area under the curve of these HA-conjugated drugs is equivalent to the AUC observed with standard systemic chemotherapies. Therefore, it does provide equivalent systemic delivery coverage for a stage 4 breast cancer in addition to providing the locoregional burst that is unique to this delivery method. Finally, regarding your last question, we looked at cisplatin and doxorubicin in the past each as single drug nanoconjugate treatments in previous papers. This research was designed to look at the combination therapy effects using nanoconjugated doxorubicin and cisplatin together against the tumor and compare it to systemic multidrug combination therapy. There were no overwhelming difficulties noted in treating the mice in combination and we observed that subcutaneous delivery system decreased even local toxicity from the doxorubicin. In fact, we did not observe any skin or subcutaneous toxicity of HA-doxorubicin delivered subcutaneously in our mice. So, that is one of the ways we looked at toxicity and we haven't had any challenges to speak of for that.

Dr William Fry (Roanoke, VA): I was just wondering whether obviously in this model, there was no interruption of lymphatics, but if this does progress to human trials, how do you foresee this being impacted by sentinel lymph node or axillary dissection as there is the potential for not being able to deliver the drug? You have interrupted the pathways for all the places that it might need to go.

Dr Cohen: The drug is delivered peritumorally as a subcutaneous injection and by doing so, it is able to attack the primary tumor. With regards to the lymphatic penetration, systemic chemotherapy is able to do that as well as we could give injections at the sites, if there was lymph node disease to further target it because of its subcutaneous delivery method, you'd be able to give the drug where you need to if there was evidence of lymphatic disease.

Dr Courtney Scaife (Salt Lake City, UT): A follow-up to that question. The advantage of nanodelivery systems is that it takes advantage of the larger gap junctions in the epithelial linings of tumors so you can deliver it systemically. Why are you not using intravenous injection with this drug?

Dr Cohen: The reason we don't use the intravenous injections of the drug is that the particle size is optimized for lymphatic uptake. When given systemically, the nanoconjugate is deposited in the liver and cleared more rapidly than when it travels lymphatically and elutes in a sustained-release format into the systemic circulation. Additionally, systemic delivery would decrease the enhanced lymphatic penetration of the drug that is achieved with subcutaneous or peritumoral delivery. Finally, a systemic delivery of drug would lead to a larger amount of drug being released from the carrier into the circulation at that initial bolus time point of infusion and this could result in a toxicity profile that is more similar to that of standard systemic delivery methods. The benefit of a subcutaneous injection and delivery is that you do not need to deal with the morbidity or resource burdens of hooking a patient up to an intravenous infusion.