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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. We hypothesize that nanocarrier-conjugated doxorubicin and cisplatin will 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 +cisplatin) at 50% or 75% MTD, and monitored for efficacy and toxicity over 12-weeks.

Results—Efficacy results for mice treated with HA-conjugated doxorubicin/cisplatin at 50% MTD include:[complete responses(CR)=5, partial responses(PR)=2, and stable disease(SD)=1]and for HA-conjugated dox/cis at 75% MTD:[CR=7,PR=1; all CR's confirmed histologically]. In comparison, mice given standard dox/cis(50% MTD)demonstrated:[progressive disease(PD)=6, SD=1, and PR=1] and for standard dox/cis(75% MTD):[PD=5,SD=3; p<0.0001 on multivariate ANOVA]. At 75% MTD, standard drug-treated mice had significant weight loss compared to nanocarrier drug-treated mice(p<0.001).

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

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Keywords

Nanocarriers; Drug-delivery; Combination chemotherapy; Hyaluronic acid; Breast cancer; Locoregional therapy

Background

Breast cancer accounted for over 209,000 new cases in 2010 with over 42,000 deaths in the United States alone, making it the leading cause of cancer in women (excluding skin cancers) and the second leading cause of cancer death in women after lung cancer (1). Although current treatments often carry an excellent short-term prognosis, up to 13% of women will develop a 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, over 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 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 to 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 first line agent is doxorubicin, which is from a class of drugs called anthracyclines. Doxorubicin decreases cancer growth by inhibiting DNA 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 a year after 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 DNA 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, over 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 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 due to 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 hyaluronan-doxorubicin (HA-dox) and hyaluronan-cisplatin (HA-cis) in breast cancer models. These studies have shown improved delivery of the chemotherapeutic agent to the lymphatic system with a decreased toxicity profile compared to the standard agent at all doses tested including lower drug doses (12, 13).

We hypothesize that combination therapy with doxorubicin and cisplatin when conjugated to nanoscopic hyaluronan (HA) as a drug-delivery carrier to the locoregional tissues and lymphatics will have improved efficacy at significantly lower dose with better lymphatic penetration and a markedly reduced toxic profile then standard combination therapy with these drugs. The aim of this study is 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.

Materials and 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 alpha (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 0.4 mg/mL G418 (Sigma Aldrich). Adherent monolayer cultures were maintained in T-75 culture flasks and incubated at 37 °C with 5% CO₂ until they achieved 85% confluency. The cells were trypsinized using 0.25% trypsin (Sigma Aldrich) and passaged into T-75 flasks at a density of 1×10⁶ 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 and coworkers (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× PBS solution at a concentration of 1×10⁶ cells/100 μL. Cells (100 μL) were injected under isoflurane anesthesia into the right first breast mound (abdominal mammary fat pad) of 4–6 week old female Nu/Nu mice using a 25G needle (20–25g, Charles River Laboratories, Wilmington, MA). Tumor size was measured 3 times weekly using a digital caliper and confirmed by two separate observers. Tumor volume was calculated using the following equation: tumor

volume (mm^3) = $(\pi/6) \times (\text{width})^2 \times \text{length}$. When tumors reached a minimum volume of 30 mm^3 , mice were randomized into control (PBS or HA) or one of four combination treatment groups [50% Maximum Tolerated Dose (MTD) Doxorubicin + 50% MTD Cisplatin (Dox-Cis 50), 75% MTD Doxorubicin + 75% MTD Cisplatin (Dox-Cis 75), 50% MTD HA-Doxorubicin + 50% MTD HA-Cisplatin (HA-Dox-Cis 50), and 75% MTD HA-Doxorubicin + 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 (s.q.) 1–3 mm away from the site of tumor implantation and the PBS control and standard treatment groups were administered intraperitoneally (i.p.). The MTD level reported in mice for doxorubicin is 8–10 mg/kg/weekly i.p. dose and for cisplatin is approximately 10 mg/kg/weekly i.p. dose (15, 16). All treatments were given 1×/week for a total of 3 weeks and mice were monitored for an additional 9 weeks upon completion of treatment (total study period of 12 weeks). Mice were euthanized prior to completion of the experiment if the tumor reached > 20 mm in diameter, if weight loss was significant or if body score markedly deteriorated.

Pathology Studies

Two Nu/Nu mice from each of the treatment groups were euthanized 1 week after 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 prior to slide mounting. Mounting using hematoxylin and eosin staining was conducted by the University of Kansas Medical Center Department of Pathology (Kansas City, KS) 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 two or more means were determined by Student's unpaired t-test (2 means) and Fisher's exact test. Multivariate analysis was performed by 2-way ANOVA followed by Duncan's multiple range test (2+ means) and Bonferroni post-hoc testing using a statistical analysis software package (SPSS version 17.0; SPSS Inc, Chicago, IL). Significance was defined for $p < 0.05$.

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 1200 mm^3 by six weeks post inoculation (Figure 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 anti-tumor activity. These groups were combined as a composite control curve (Figure 1). Of the experimental 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 a complete response (CR) and the remaining mouse having a partial response (PR) with 87% reduction in tumor volume (Figure 1). The second best group for efficacy was the HA-Dox-Cis 50 group, where 7 of 8 mice (87.5%) had a significant response to treatment (5 CRs and 2 PRs), with the remaining mouse having stable disease [(SD), Figure 1]. Alternatively, in the standard treatment groups at comparison MTD levels, the Dox-Cis 75 group had only 2 of 8 (25%) animals with a partial response 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 PR (12.5% response rate), one animal with stable disease, with the remaining 6 animals (75%) having progressive disease (PD, Figure 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 CRs were true pathologic complete responses. An overall comparison of all 4 treatment groups using a multivariate analysis was noted to be statistically significant at $p < 0.0001$ and when breaking this down to compare individual groups, the response rate among the HA-Dox-Cis 50 group compared to the standard 50 group and the standard 75 group was noted to be statistically significant ($p = 0.0004$ and $p = 0.005$, respectively). Conversely, comparing the HA-Dox-Cis 75 group to the standard 50 and 75 groups was also noted to be statistically significant ($p < 0.0001$ and $p = 0.0003$, respectively). Of note, comparison between the 2 doses levels of the standard treatment was not noted to be statistically significant ($p = 0.27$).

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 (Figure 2A) compared to the visible tumors in the standard Dox-Cis 50 and Dox-Cis 75 groups (Figure 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. Upon histological examination, both HA treatment dosing groups showed fibrosis and neutrophil infiltration but no histologic evidence of residual tumor at the tumor site (Figure 2C) compared to the standard treatment at both doses, which had residual tumor with associated central necrosis (Figure 2D). Additionally, there was no evidence of lymph node metastases present in any of the treated animals while over 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 kidney in any of the treated groups. Systemic disease was however noted histologically as spinal metastases in one of the mice at the 50% MTD systemic Dox-Cis 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 of 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 ($p = 0.4917$). In comparing the weight loss profiles of the HA groups to dose-matched standard drug combinations, it was noted that there was no weight loss noted in the standard 50% group as well, however, 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 ($p < 0.001$). (Figure 3A). It should be noted, though, that the HA-Dox-Cis-75 group did demonstrate some weight loss (average of 10%) while receiving the 3 weeks of treatment, however 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 due to this toxicity, particularly in the Dox-Cis-75 group, where 5 animals were sacrificed for clinical toxicity prior to completion of the study (Figure 3B).

Discussion

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 over 30% of patients who also receive radiation and in 10–20% 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 due in part to 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 hyaluronan. We have reported that in vivo usage 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 to the standard drug formulations (12, 13). In practice, however, chemotherapy for LABC 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 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 following 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 due to 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 AUC levels 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 to standard agents. Those studies demonstrated comparable levels of systemic penetration via equivalent plasma AUC levels (12,13). Intratumoral as well as lymphatic levels of HA-cisplatin compared to systemic cisplatin were also measured demonstrating significantly increased levels of cisplatin in the tumor and lymphatic tissues in the HA-Cis group compared to systemically delivered cisplatin (13).

While previous studies have demonstrated improved efficacy and pharmacokinetic 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 due to synergistic effects. Therefore, combination of two nanoconjugated agents *in vivo* would be expected to further enhance this synergy. Although individual uptake of each drug was not measured intra-tumorally in the combination therapy, based on the dramatically improved efficacy of the combination nanoconjugated agents compared to 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, due to 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 70% 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 pathological 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 a significantly improved efficacy for the nanocarrier delivered drug combination even at half of the standard dose of current therapy.

With regards 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 prior to study-completion due to significant clinical toxicity as evidenced by decreased body score 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 three doses of drug are given, all at 75% or less 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-combinational treatment.

Overall, we conclude that based on 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 to 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 the 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 one drug at a time as per FDA regulations for safety and efficacy in patients prior to any combinational therapy. To 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 LABC patient population. While 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 (AUC) 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 the 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 prior to surgical

resection in hopes to prevent future recurrence or in patients who have locoregional recurrence and have failed traditional systemic agents or are limited in the administration of these agents to due 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 for 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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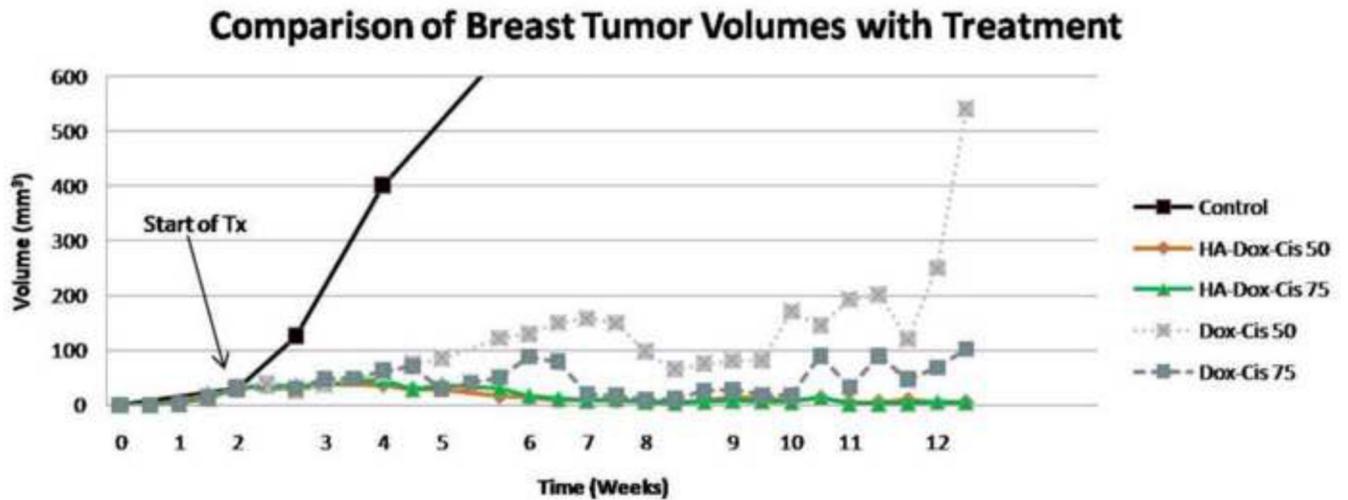


Figure 1.

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 s.q. injection and 1x PBS systemic injection (N=4 for each). Note, there is delay in tumor growth with standard Dox-Cis treatment, however, progressive disease does still occur whereas there were significantly more complete responders in the HA treatment groups, which was durable.

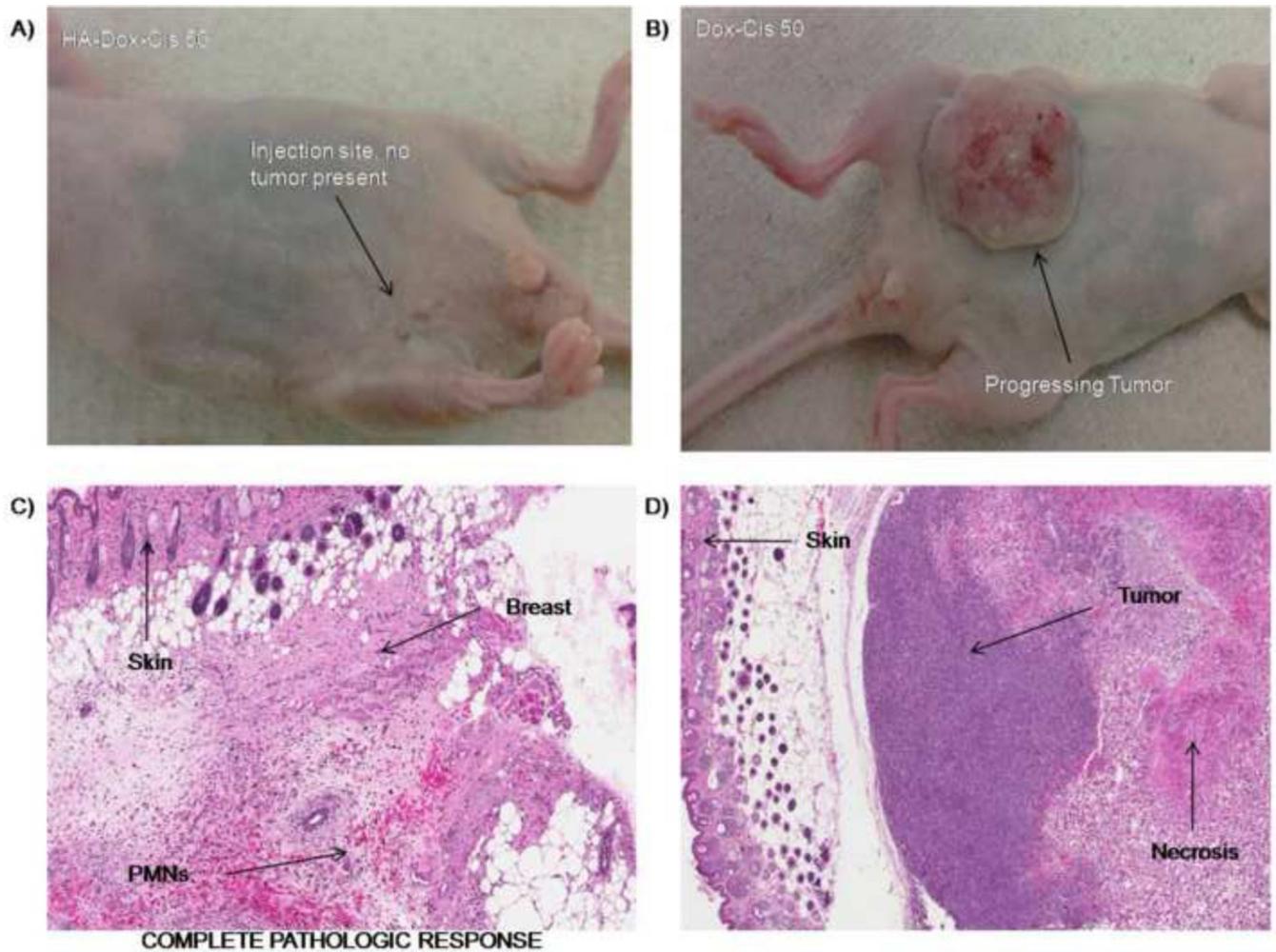


Figure 2.

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 following treatment. C) Hematoxylin and eosin stained histologic image at 7.8 \times magnification of mouse from image 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 pathological response. D) Hematoxylin and eosin stained histologic image at 5.4 \times magnification of mouse treated with Dox-Cis 50 demonstrating a partial response clinically. Arrows denote skin, histological presence of tumor with associated central necrosis.

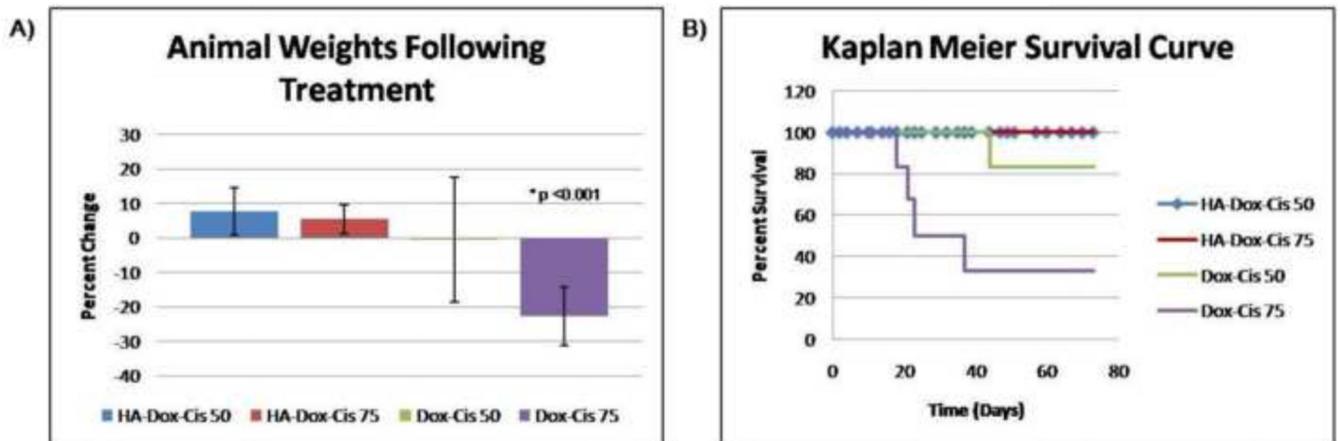


Figure 3.

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 no durable weight loss in the HA-Dox-Cis groups, which was statistically significant ($p < 0.001$). B) Kaplan-Meier Survival Curves by Group. Note, both HA-Dox-Cis groups had 100% survival throughout the study, which was superior to the Dox-Cis groups. $N=6$ for each group as 2 animals were euthanized immediately following treatment for histology. Note, controls were all euthanized by week 7 due to advanced tumor volumes and deteriorating body condition from progressive disease per established animal protocol endpoints, and therefore were not included in figures 3A and 3B.