Intralymphatic Chemotherapy Using a Hyaluronan–Cisplatin Conjugate

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Background. Breast cancers typically spread to regional lymph nodes once they disseminate from the primary tumor, thus adequate evaluation and treatment of the axillary lymph nodes is paramount in early stage disease. One significant problem with current therapy is the side effects chemotherapy agents create systemically, either alone or in combination. The purpose of this study is to determine whether lymphatically targeted cisplatin carriers will increase the localized dose in lymphatic metastases without systemic toxicities.

Methods. Hyaluronan (HA) is a highly biocompatible polymer that follows lymphatic drainage from the interstitial spaces. We formed complexes of HA and cisplatin by non-covalent conjugation. Complexes were injected subcutaneously into the upper mammary fat pad of female rats, and the tissue distribution determined.

Results. Cisplatin–HA contained up to 0.25 w/w of Pt and released drug with a half-life of 10 h in saline. Cisplatin–HA conjugates had high anti-tumor activity *in vitro* similar to the free drug: cisplatin–HA IC$_{50}$ 7 μg/mL in MCF7 and MDA-MB-231 human breast cancer cells (free cisplatin IC$_{50}$ 7 μg/mL). Cisplatin–HA conjugates were well tolerated in rodents with no signs of injection site morbidity or major organ toxicity after 96 h. The area-under-the-curve of cisplatin in the axially lymph nodes after injection with cisplatin–HA increased 74% compared with normal cisplatin.

Conclusions. This study demonstrates a novel intralymphatic drug delivery method in breast cancer to preferentially treat at-risk regional lymph nodes and avoid systemic toxicities. Further *in vivo* testing related to efficacy of this approach with regard to survival, toxicity, and pharmacokinetics is warranted to support its use in human trials. © 2008 Elsevier Inc. All rights reserved.

Key Words: cancer; polymeric drug carrier; lymphatic transport; cancer chemotherapy.

INTRODUCTION

Early breast cancers spread initially from the primary tumor site to regional lymph nodes in the axilla prior to systemic dissemination. Surgery and radiation therapy can be effective for loco-regional disease, but carry a moderate morbidity risk including painful lymphedema [1]. We therefore sought to develop chemotherapeutic formulation that could be given locally and isolated to the draining lymphatic basin of the breast, where early metastases are more prevalent. Cisplatin is rarely used in routine treatment of breast cancer, but may be useful in combination therapy as several recent studies have demonstrated the efficacy of platinum combinations with taxanes and trastuzumab [2].

The inclusion of platinums in regimens has been associated with several toxicities, including increased risk of leukopenia, nausea, hair loss, acute nephrotoxicity, chronic neurotoxicity, and anemia [3]. Thus, a loco-regional therapy approach for disease confined to the breast and axilla may greatly improve the use of platinums in breast cancer chemotherapy. For this purpose, hyaluronan may be an ideal carrier for localizing cisplatin to the lymph nodes. Hyaluronan (HA) is a polysaccharide, of alternating D-glucuronic acid and *N*-acetyl D-glucosamine, found in the connective tissues of the body and cleared primarily by the lymphatic system (12 to 72 h turnover half-life [4]). After entering

the lymphatic vessel, HA is transported to nodes where it is catabolized by receptor-mediated endocytosis and lysosomal degradation. Several studies have correlated increased HA synthesis and uptake with cancer progression and metastatic potential [5, 6]. Breast cancer cells are known to have greater uptake of HA than normal tissues [7], requiring HA for high P-glycoprotein expression, the primary contributor to multi-drug resistance [8]. Knockout of HA receptors has been reported to prevent migration of cancers that initially spread intralymphatically [9]. Furthermore, invasive breast cancer cells overexpress CD44, the primary receptor for HA [10], and are dependent on high concentrations of CD44-internalized HA for proliferation (reviewed in [7]). Cisplatin conjugates to HA may be very efficacious against lymphatic metastases.

Previous reports have demostrated the ability of HA to form stable conjugates with platinum drugs [11, 12], but this is the first *in vivo* study of cisplatin–HA and the first examination of HA-drug conjugates designed for lymphatic deposition and retention. Furthermore, we show that HA is drained to the axilla basin of rats after subcutaneous injection into the mammary fatpad, laying the foundation for future studies of tissue distribution, pharmacokinetics, and anti-tumor activity in rodent models of breast cancer.

MATERIALS AND METHODS

Materials

Hyaluronan from microbial fermentation was purchased from Lifecore Biomedical (Chaska, MN) as sodium hyaluronate and used without further purification. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA) and were of ACS grade or better. Milli-Q water was used in all experiments. Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained according to ATCC recommendations. Animal procedures were approved by the University of Kansas Animal Care and Use
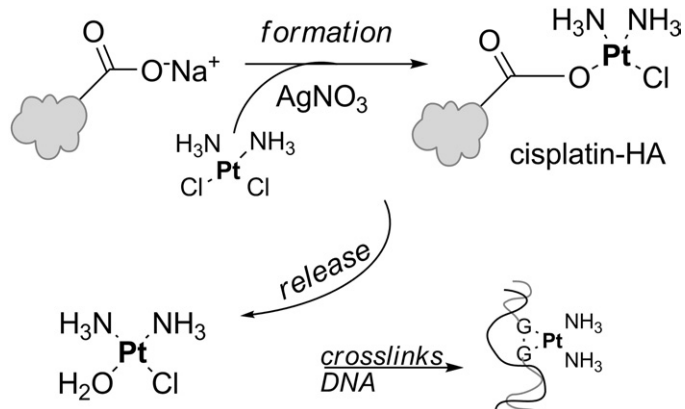
TABLE 1

Conjugation Efficiency of Cisplatin to HA

Cisplatin added w/w cisplatin/HA	Conjugated w/w cisplatin/HA	Conjugation efficiency (%)*
0.03	0.022	73
0.08	0.040	50
0.15	0.086	57
0.20	0.119	60
0.30	0.149	50
0.40	0.210	53
0.50	0.254	51
0.60	0.263	44
0.70	0.241	34

* Efficiency calculated as (cisplatin added/cisplatin incorporated) $\times$ 100%.

committee. **Caution:** Cisplatin is extremely toxic and all chemical waste and animal tissues were treated as hazardous waste and disposed of accordingly.

Synthesis of Cisplatin-HA Conjugates

Cisplatin was conjugated to HA (35,000 g/mol) using silver nitrate as an activating reagent [12]. HA (100 mg), cisplatin (45 mg), and silver nitrate (25.5 mg) were dissolved in H_2O (20 mL) and stirred in the dark for 18 h at ambient temperature (ca. 25°C). The reaction mixture was filtered (0.2-μm nylon membrane) and dialyzed against H_2O (3500 MWCO; Pierce, Rockford, IL) for 48 h at 4°C. Following dialysis, the crude product was concentrated and stored as a 9.1-mg/mL solution, and the degree of cisplatin substitution was determined by atomic absorption spectroscopy (AAS). The resulting conjugate is referred to as cisplatin–HA for clarity, although the conjugate is $[PtCl(H_2O)(NH_3)_2]OOCO$-HA (Fig. 1). During the course of completing this study, a similar procedure for conjugation to high molecular weight HA was reported by Jeong *et al.* [11].

Fluorescent conjugates of HA were formed by condensation of Texas Red hydrazide to HA. HA (35,000 MW, 100 mg) in 10 mL of 30% H_2O:Ethanol was activated with 2-chloro-1-methylpyridinim iodide (33 mg) and triethylamine (35 μL). After the addition of Texas Red hydrazide (AnaSpec Inc., San Jose, CA; 2 mg in 0.4 mL of

FIG. 1. Cisplatin conjugated to HA nanocarrier carboxylate. HA releases *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$, which effects anticancer activity by crosslinking DNA.
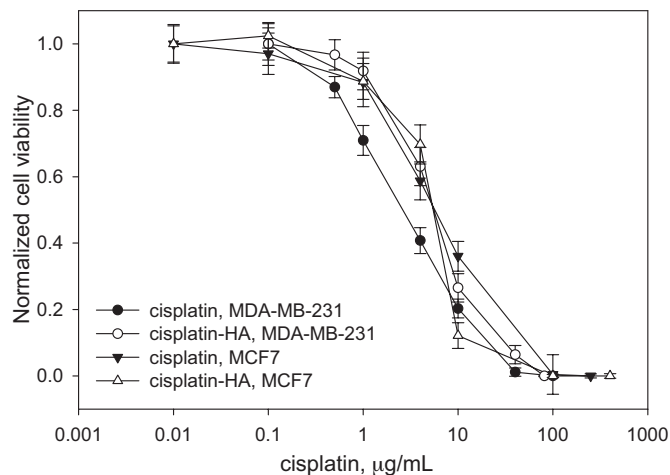

FIG. 2. Inhibition of human cancer cell growth by cisplatin and cisplatin–HA after 72 h. HA showed no toxicity over the examined concentrations (up to 10 mg/mL; data not shown).
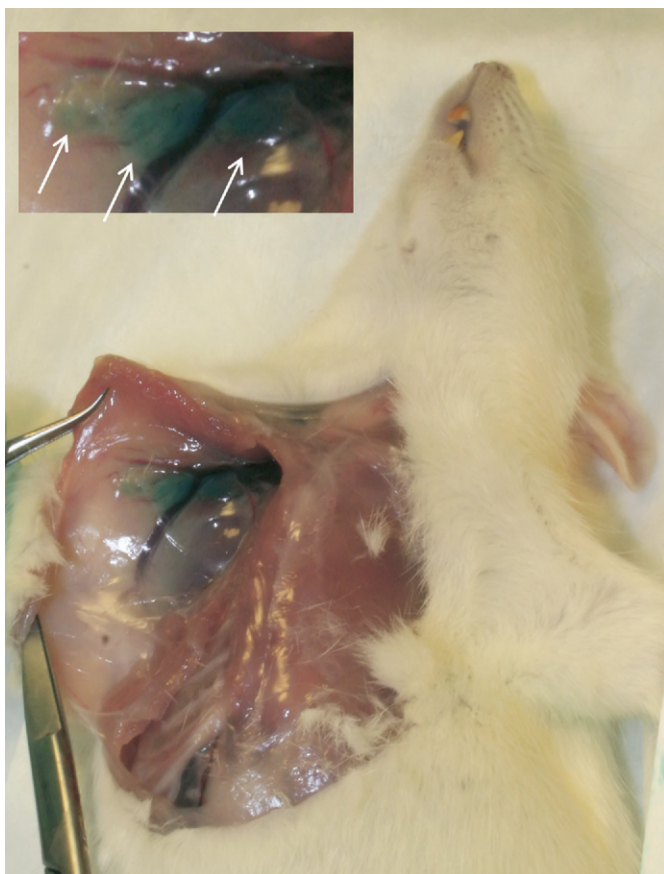
FIG. 3. The axial lymph node package was excised after euthanizing animals (white arrows). To identify draining lymph nodes, 100 μL of patent blue was injected subcutaneously into the fatpad and the animal euthanized after 5 min (patent blue was not used in distribution studies).

dimethyl sulfoxide), the mixture was refluxed for 24 h. Work-up proceeded by dialysis against H_2O for 48 h at ambient temperature, followed by lyophilization. Conjugation efficiency was determined by using a molar extinction coefficient of 81,800 $M^{-1}cm^{-1}$ at λ 588 nm.

In Vitro Drug Release

In vitro release rate of cisplatin from cisplatin–HA was determined in phosphate buffer with and without saline. Cisplatin–HA was added to 3500 MWCO dialysis bag (Pierce) and placed in a phosphate-buffered water bath (pH 7.4, 37°C) or physiological saline (140 mM). Samples were taken from the dialysis bags at predetermined time points, and remaining Pt concentration was determined by AAS.

Cell Toxicity

Cell lines were seeded into 96-well plates (5000 cells/well) in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. After 24 h, cisplatin, cisplatin–HA, or HA was applied ($n = 12$; 7 concentrations), and 72 h post-addition, reazurin blue in 10 μL phosphate-buffered saline was applied to each well (final concentration of 5 mM). After 4 h, well fluorescence was measured (λ_{ex} 560 nm, λ_{em} 590 nm; SpectraMax Gemini; Molecular Devices, Sunnyvale, CA), and the IC_{50} determined as the midpoint between saline (positive) and cell-free (negative) controls.

Tissue Distribution

Sprague Dawley rats (200-250 g females; Charles River Laboratories, Wilmington, MA) were placed under isoflurane anesthesia and injected subcutaneously (100 μL) into the right mammary fat pad with cisplatin–HA or cisplatin in 0.9% saline (3.5 mg/kg equivalent cisplatin; $n = 5$). Animals were allowed to recover with access to food and water. After 1, 4, 12, 24, 48, and 96 h post-injection, animals were euthanized by isoflurane overdose. Organs and tissues were washed with 0.9% saline and frozen (-80°C) until analysis. Plasma was separated by centrifugation from whole blood and frozen (-80°C).

Atomic Absorption Spectroscopy for Cisplatin (AAS)

In vitro release samples ($n = 3$) and plasma samples ($n = 5$) were diluted 200-fold and 10-fold, respectively, with 0.1% nitric acid for analysis. Tissue samples (except for lymph nodes) were prepared by digesting 50 mg of tissue in 1.5 mL of 6.7% nitric acid for 1 h at 80°C. Lymph nodes were processed similarly using 10 mg of tissue. After digestion, samples were homogenized (Tissue Tearor; BioSpec Products Inc., Bartlesville, OK). All samples were centrifuged ($17,000 \times g$, 20 min), and the supernatant was used for analysis.

Analysis was performed on a Varian SpectrAA GTA-110 with graphite furnace and partition tubes. Samples (21 μL) were injected using the autosampler, followed by 19 μL of 0.1% nitric acid. Every 10 samples were bracked by calibration standards at 150, 300, and 450 ng/mL, and a quality control sample (150 or 300 ng/mL) every 5 samples. A full calibration curve was prepared from 1 to 450 ng/mL in 0.1% nitric acid (10 concentrations). Pt recovery was determined by spiking tissue blanks with cisplatin or cisplatin–HA (50 μg/g) and processing as above. The furnace program was as follows: ramp 25 to 80°C, hold 2 s, ramp to 120°C, hold 10 s, ramp to 1000°C, hold 5 s, ramp to 2700°C, hold 2 s, cool to 25°C over 20 s. The graphite partition tube was cleaned every 40 samples by baking at 2800°C for 7 s. Argon was used as the injection and carrier gas.
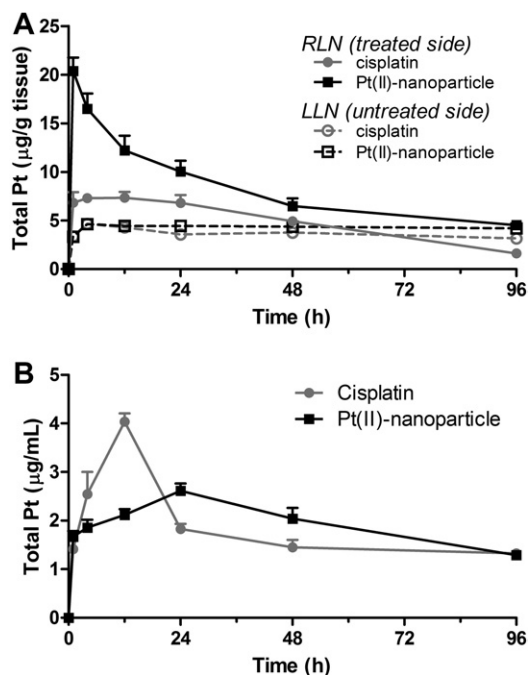

FIG. 4. (A) Tissue concentration of Pt in right axilla lymph nodes (RLN) and left axilla lymph nodes (LLN) after subcutaneous injection of cisplatin or cisplatin–HA (3.5 mg/kg Pt basis) into right mammary fatpad. (B) Plasma concentration of Pt.

TABLE 2

Tissue AUC of Cisplatin and Cisplatin–HA

	AUC* (0-96 hrs), $\mu g \cdot hr/g$	
Tissue	Cisplatin	Cisplatin–HA
Bladder	208.5	194.5
Brain	442.5	343.7
Heart	459.0	465.3
Kidneys	650.9	668.6
Liver	415.6	495.0
Lungs	409.8	347.3
Muscle	371.5	262.1
Spleen	349.1	383.4
Left lymph node	349.5	413.4
Right lymph node	446.0†	776.0†
Plasma	174.1	186.1

* AUC of Pt after subcutaneous administration of cisplatin or cisplatin–HA into the right mammary fatpad of female rats ($n = 5$). Significance determined by 2-way analysis of variance with replicates.
  † $P < 0.05$.

In Vivo Imaging

Lymphatic breast tumor metastasis was induced in nude mice according to the procedure of Chambers and coworkers [13], who were kind enough to provide the lymphatically metastatic breast tumor cell line MDA-MB-468LN. Nude mice (25-30 g females; Charles River) were anesthesized with pentobarbital (50 mg/kg), and 100 μL of MDA-MB-468LN (10^7 cells/ml) was injected orthotopically into the left second thoracic mammary fatpad through a small incision later closed with a wound clip. Tumors were palpable after 4-5 wks (100-300 mm^3). Before imaging, mice were anesthesized, and Texas Red–HA (10 mg/mL in saline, 20 μL) was injected subcutaneously over the left mammary fatpad. The injection area was massaged gently for 5 min and fluorescently imaged after 5 and 18 h (CRI Maestro Flex; CRI Inc., Woburn, MA) using a 445- to 490-nm filtered halogen excitation light and a 515-nm longpass emission filter. Fluorescence was measured in 10-nm bandpass segments from 520 to 720 nm, using a cooled CCD camera with autoexposure. Images were spectrally unmixed by using the automatic deconvolution tools (Maestro version 2.4) to limit skin and intestine autofluorescence resulting from chlorophyll in food.

RESULTS

Cisplatin–HA Conjugation, Release, and Measurement

Cisplatin was highly conjugated to HA, with typical conjugations of 0.25 w/w cisplatin/complex using a starting ratio of 0.5 w/w cisplatin/HA. Up to 0.75 w/w cisplatin/complex was attempted with decreasing efficiency (Table 1). The release rate of cisplatin from complexes was determined in both phosphate-buffered saline and water. The Cl$^-$ in saline was expected to more rapidly displace cisplatin, increasing the release rate. The release of drug showed near first order release kinetics with a release half-life of 42 h in water and 10 h in physiological saline. The AAS produced a linear concentration curve from 10 to 450 ng/mL ($R^2 = 0.9998$), with a limit of detection of 5 ng/mL and a limit of quantification of 10 ng/mL (5% standard deviation). Pt recovery from cisplatin–HA-spiked tissues was: plasma, 82% ± 4% (±STD); lymph nodes, 92% ± 2%; bladder, 88% ± 1%; brain, 94 ± 0.3%; heart, 97% ± 1%; kidneys, 98% ± 1%; liver, 100% ± 1%; lung, 94% ± 1%; muscle, 95% ± 1%; spleen, 97% ± 1%. Pt recovery from cisplatin-spiked tissues was: plasma, 80% ± 3%; lymph nodes, 92% ± 6%; bladder, 86% ± 3%; brain, 93% ± 10%; heart, 93% ± 5%; kidneys, 100% ± 2%; liver, 100% ± 7%; lung, 95% ± 8%; muscle, 100% ± 5%; spleen, 96% ± 9%.

Cell Toxicity

Cisplatin–HA conjugates had similar toxicities to free cisplatin in cell culture. Toxicity was evaluated in the highly metastatic human breast cancer cell lines MCF7 and MDA-MB-231 (Fig. 2). In both cell lines, there was no appreciable difference in toxicity between cisplatin–HA (IC$_{50}$ 7 μg/mL, cisplatin basis) and cisplatin (IC$_{50}$ 7 μg/mL). HA had no toxicity to human cells over the concentration range examined (up to 10 mg/mL; data not shown).

Tissue Distribution

Conjugation of cisplatin to HA impacted the local concentration of cisplatin in draining lymph nodes (Fig. 3) with a minor effect on systemic concentrations (Fig. 4, Table 2). Over the experimental timeframe of 96 h, the area-under-the-curve (AUC) of cisplatin–HA conjugates in the right lymph node, which drains the
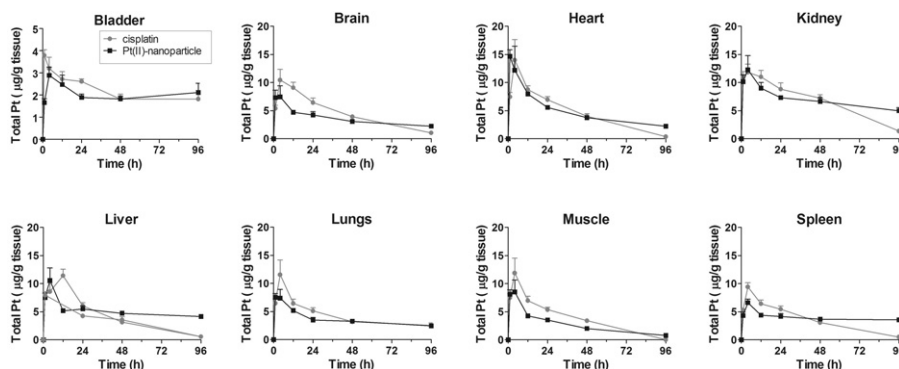

FIG. 5. Tissue concentration of Pt after subcutaneous injection of cisplatin or cisplatin–HA (10 mg/kg Pt basis) into right mammary fatpad.

injection site, was 74% greater than cisplatin in saline ($P = 0.0001$), and the right lymph node had increased tissue concentrations over the examined period (Fig. 4A). The AUC of cisplatin–HA in the non-draining left lymph node was not significantly different from cisplatin in saline ($P = 0.12$).

A burst release of free cisplatin appeared in the plasma concentration profile (Fig. 4B), whereas cisplatin–HA demonstrated a longer, sustained release into the plasma. This is signficant because dose-limiting toxicities of cisplatin therapy are strongly influenced by peak plasma concentration [14]. There was not a significant difference in the plasma AUC between cisplatin–HA and cisplatin ($P = 0.13$). Thus, localized therapy with cisplatin–HA may generate sufficient serum concentrations to treat distant metastases, while providing a boost therapy for the breast lymphatics. The distribution of Pt to other organs was not significantly different over the study period (Fig. 5, Table 2).

In Vivo Imaging

Fluorescent conjugates of HA to Texas Red (1 dye/23 HA molecules) drained into the left lymphatic basins after a subcutaneous injection into the left mammary fatpad, with no observed bilateral drainage into the right basin. After 5 h, localization of HA in the nodes surrounding the tumor can be clearly observed (Fig. 6B). Most of the carrier has localized in the basin of the tumor at 18 h with a lesser amount remaining in the adjacent axillary node basins (Fig. 6C).

DISCUSSION

This study demonstrates that intralymphatic delivery of platinum chemotherapeutics may greatly increase the local concentration in drain lymph node basin, with no determination impact on non-targeted organs or systemic concentrations. Cisplatin, although not typically given as single-agent chemotherapy in breast cancer, has been used in combination therapies [2]. One problem associated with platinum-based chemotherapies is renal toxicity. If a better method of delivering a somewhat toxic agent like cisplatin could limit organ toxicity while maintaining tumor efficacy and delivering a higher drug concentration to nodal tissue where most locoregional tumor cells reside, this would open up several more treatment strategies which have previously been abandoned. Our intralymphatic delivery model using cisplatin–HA not only increases drug concentrations in loco-regional nodal tissue significantly above the standard cisplatin formulation (74% greater AUC), but it also exhibits sustained release kinetics, allowing lower C_{max} levels which could translate into lower organ toxicity over time. Looking at tissue distribution of the drug and comparing tissue levels of cisplatin with cisplatin–HA,
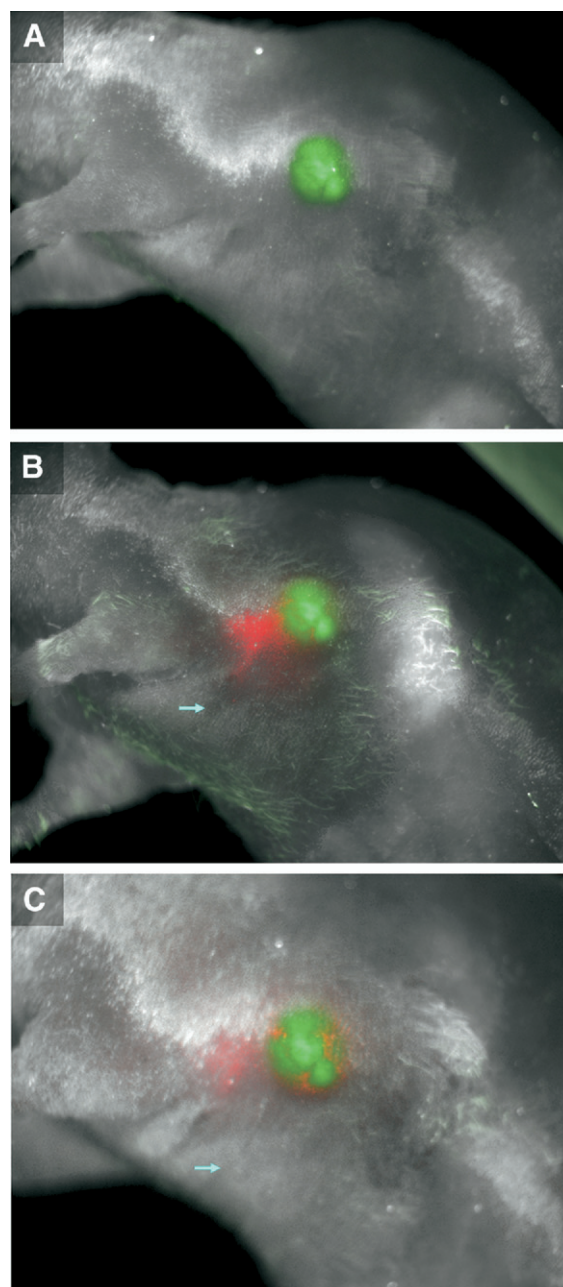

FIG. 6. Nude mice bearing MDA-MB-468 breast lymphatic tumors (expressing green fluorescent protein [GFP]; A) were subcutaneously injected with Texas Red–HA in the left mammary fat pad. After 5 and 18 h (B and C, respectively), significant HA localized in the draining nodes and colocated with the tumor (GFP channel in green, Texas Red channel in red, blue arrow is injection site).

the only tissue level that was significantly different was the axillary lymph nodes ipsilateral to the drug injection. This translated into almost double the concentration of cisplatin penetrating the loco-regional nodes using cisplatin–HA compared with cisplatin injected directly into the breast subcutaneously. This nodal level achieved by cisplatin–HA would be even more significant if one compared IV cisplatin with

peritumoral-injected cisplatin–HA, since most of IV cisplatin is cleared through the liver and kidneys and only a very small fraction of the injected dose penetrates the nodal tissue of concern. Therefore, cisplatin–HA delivered through the lymphatics would significantly boost the concentration of drug loco-regional tumor cells come in contact with. This may lead to more effective tumor reduction in these areas and possibly lower loco-regional recurrence rates clinically.

To date, this is the first successful application of intralymphatic delivery of chemotherapeutics using hyaluronan as a targeted nanocarrier to the axilla. An intralymphatic drug delivery method in breast cancer may preferentially treat at-risk regional lymph nodes and avoid systemic toxicities associated with IV or oral drug administration. Once breast cancer has spread to loco-regional nodes, it is likely to assume that tumor cells may have disseminated into the systemic circulation. In fact, for patients with sentinel nodes containing micrometastases, systemic chemotherapy is routinely given after axillary nodal dissection. We believe the benefit of this delivery model is that it not only achieves adequate systemic drug levels in a more sustained-release manner than standard therapy, but it also provides a much-needed boost to the loco-regional nodal tissue, which is at risk for harboring tumor cells not removed by nodal dissection. Additionally, this modality may have its best application in the neoadjuvant setting for locally advanced breast cancers, where the goal of disease control and regression can be enhanced by the drug concentration boost this intralymphatic delivery gives to locoregional nodes, harboring the bulk of tumor in this condition. In using this delivery method to increase locoregional efficacy, it may be possible to improve overall and disease-free survival rates compared with current therapy regimens. Further *in vivo* testing related to the efficacy of this approach with regard to survival, toxicity, and pharmacokinetics in rodents and large mammals is warranted to support its use in human trials.

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