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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

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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 at 3.3 mg/kg (platinum basis).

Results—Nanoconjugates co-localized with the tumors after subcutaneous peritumoral injection and demonstrated improved efficacy to intravenous cisplatin. After one month, renal tubular hemorrhage and edema were more prevalent in the intravenous formulation compared to subcutaneous HA-cisplatin nanoconjugates.

Conclusions—This nanocarrier delivery platform focuses drug in the areas where tumor burden is greatest, potentially reducing systemic toxicity, and has future applicability as a neoadjuvant or adjuvant therapy for LABC.

SUMMARY

Locoregional delivery of cisplatin to the breast and axilla using a subcutaneously injected hyaluronic acid nanoconjugate demonstrated improved efficacy and decreased toxicity compared to intravenous cisplatin in a murine orthotopic model of locally advanced breast cancer and may hold promise for future clinical studies.

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].

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Neoadjuvant chemotherapy is considered standard of care for locally advanced breast cancer (LABC), given to decrease 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. [4] 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.

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[6]. Additionally, most patients (as high as 75% to 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 (e.g. daily), have been shown to significantly reduce nephrotoxicity and ototoxicity [9, 10] due to lower peak plasma concentration, but metronomic dosing requires more frequent treatments, 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 [11, 12]. 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[13].

MATERIALS AND METHODS

Nanoconjugate synthesis

Hyaluronan-cisplatin(HA-Pt) nanoconjugates were formed by stirring 10% weight/volume (w/v) hyaluronan (HA,; 35 kDa; 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 kDa cellulose tubing, Pierce, Rockford, IL) for 48 hrs at 4°C, lyophilized, and CDDP conjugation was determined by atomic absorption spectroscopy (Varian SpectAA GTA-110). Nanoconjugates typically contained 25% weight/weight 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 0.4 mg/ml G418.[13,16] Preceding proliferation studies, cells were trypsinized and seeded into 96-well plates (5,000 cells/well). After 24 hrs, CDDP, HA-Pt (with or without silver activation), or HA was added (n=12; 7 concentrations), and 72 hrs post-addition, resazurin blue in 10 μl of phosphate-buffered saline was added to each well (final concentration of 5 mM). After 4 hrs, well fluorescence was measured (λ_{ex} 560 nm, λ_{em} 590 nm) using a fluorophotometer (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA). IC_{50} was determined as the midpoint between saline (positive) and cell-free (negative) controls for each plate. For comparison, two 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 two groups and administered CDDP intravenously via the tail vein or HA-Pt subcutaneously into the mammary fat pad (1.0 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 haematoxylin & eosin (H&E) staining were conducted by Veterinary Lab 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 (0.25% w/v trypsin) and prepared in 1×PBS solution at three different cell concentrations (10^5, 10^6, 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-ga 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 (GFP), 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 using equation: tumor volume (mm^3) = $0.52\times(\text{width})^2\times\text{length}$. Animals were euthanized before the study's end when their tumor size reached 2000 mm^3 or the body score index fell under 2. Tumors of 50 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

Prior to 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, since one or more of the chlorides can be displaced allowing formation of a labile ester linkage with the polymer [17]. CDDP was highly conjugated to hyaluronan, with typical nanoconjugates having 25% weight/weight 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 hrs. HA-Pt nanoconjugates had similar cytotoxicity (IC50)in vitro to the standard CDDPformulation in multiple breast cancer cell lines tested: MDA-MB-468LN, 3.9 and 3.6 μM (CDDP, HA-Pt);MDA-MB-231, 5.9 and 5.9 μM; and MCF-7, 5.7 and 5.2μM. HA showed no toxicity at 10 mg/ml, the upper limit of testing (data not shown).

In vivo efficacy analysis in xenografts

Control animals demonstrated a standard tumor growth curve at 10^6 cells/injection with tumor volumes exceeding 1000 mm^3 at 6 weeks post-inoculation (Figure 1A). HA carrier-only animals demonstrated no difference from controls confirming in vitro data that HA has no direct anti-cancer activity. The intravenous standard CDDP-treated animals demonstrated a tumor-growth delay of about 3 weeks compared with controls ($p < 0.05$) with a median survival of 12 weeks (compared to 7 weeks in controls $p<0.01$; Figure 1B). HA-Pt treated animals had an initial delay in tumor growth of 5 weeks ($p<0.01$ compared to controls but this was NS compared to i.v. CDDP with both curves meeting by 12 weeks post-inoculation) with a median survival of 12 weeks as well. However there was one animal in the HA-Pt group who demonstrated 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 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 i.v. CDDP (3.3 mg/kg) and s.c. HA-Pt. Very mild changes were observed in the livers for animals receiving both low dose CDDPi.v. (1.0 mg/kg) and low dose HA-Pt s.c. indicated by the presence of mild inflammation in the sinusoids (Figure 1C). Mild degeneration with some sinusoidal necroses were observed for animals receiving high dose i.v. CDDP and high dose s.c. HA-Pt treatment. Necroses, however, were more severe in the i.v.CDDP group. In addition, 60% of animals receiving low dose i.v.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 s.c. HA-Pthad renal tubular necrosis. Similarly, 4 of 5 (80%) animals receiving high dose i.v. CDDP compared to 1 of 5 (20%) animals receiving high dose s.c. HA-Pt were diagnosed with mild renal tubular necrosis. Overall, the pathology studies demonstrated that the HA-Pt conjugates had lower incidence of both renal and hepatic toxicity compared to the conventional i.v.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).

DISCUSSION

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 30–50% of patients[18–20]. Cytotoxic chemotherapies also have poor penetration to the locoregional lymphatics in the breast due to separation of the lymphatics from the systemic vasculature as well as lymphatic mono-directional flow [21]. 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 increasing evidence that these patients may have increased platinum sensitivity[22]. Recent studies report that BRCA1 breast cancers are highly sensitive to platinums due to the role BRCA1 plans in DNA double-strand repair [23–24].

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.[13] Nanoconjugation of CDDPto hyaluronic acid not only allows for improved locoregional delivery of drug to the site of greatest tumor burden in the breast and axillary tissues, but also decreases the level of renal toxicity associated with this drug. Our toxicity data demonstrate that there is no significant injection site toxicity on pathologic 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 to 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 vs. CDDP tumor-growth curves in Figure 1A demonstrate 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 week additional delay compared to CDDP and 5–6 week delay compared to 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 (Figure 1A–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 since 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 provides promise for future potential use in LABC in both the neoadjuvant or adjuvant setting. In addition, localized therapy may 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 demonstrated that HA-Pt given locoregionally provides adequate systemic levels of CDDP including serum AUC levels greater than intravenous CDDP but without the high (toxic) peak serum concentrations of intravenous therapy[13]. 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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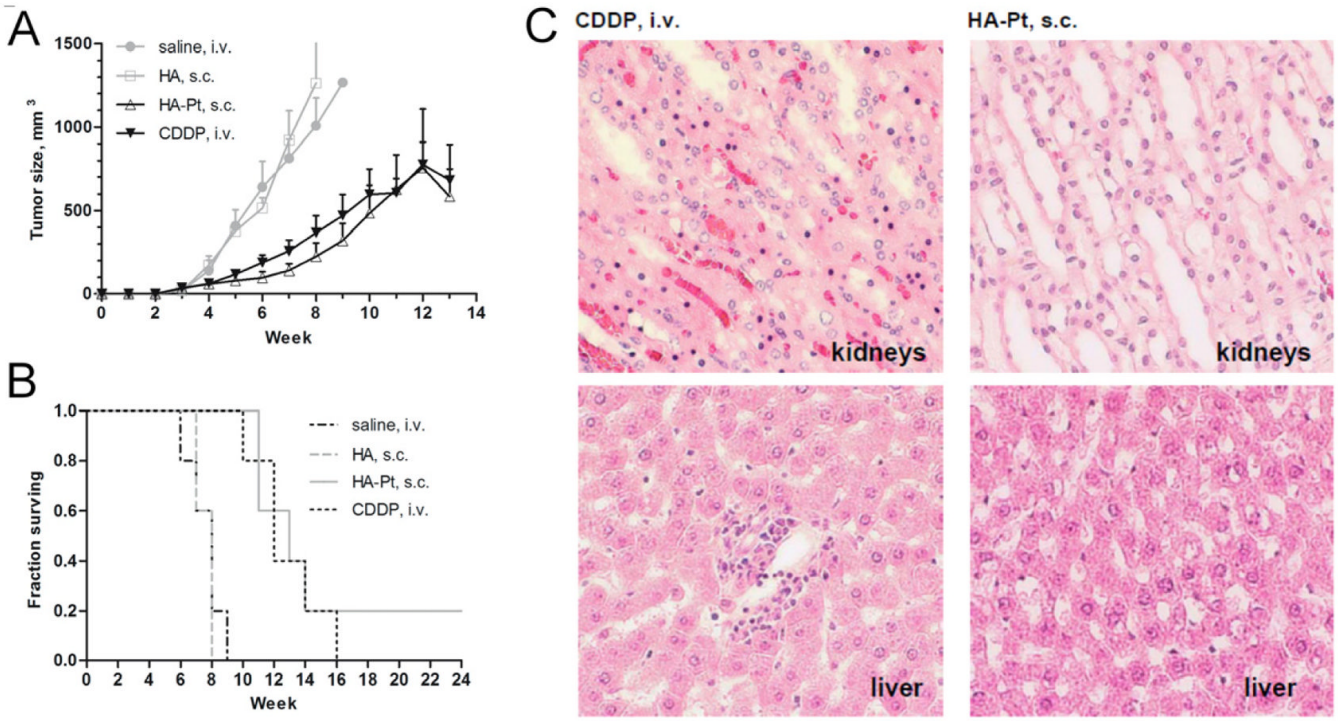

Figure 1.

(A) Measurement of tumor size. 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 1000 mm^3 and no tumor ulceration or infection (n=5). **(C)**Kidneys of the s.c. HA-Pt group had a normal apperance except for sparse minimal tubular cell necrosis; whereas, the i.v. CDDP treatment group had pyknotic nuclei and apoptosis in the medullary tubular epithelia cells. Livers of the s.c. HA-Pt group had very minor hepatitis but otherwise appeared normal; whereas, the i.v. 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).