

In Vivo Efficacy of Nano Hyaluronan-Conjugated Cisplatin for Treatment of Murine Melanoma

QiuHong Yang MS,^{a*} Daniel J. Aires MD JD,^{b*} Shuang Cai PhD,^a Garth R. Fraga MD,^b Da Zhang MD,^b Cicy Z. Li MS,^b and M. Laird Forrest PhD^a

^aUniversity of Kansas, Lawrence, KS

^bUniversity of Kansas Medical Center, Kansas City, KS

*Contributed equally to this article

ABSTRACT

Background: Melanoma is a deadly skin cancer with rapidly rising incidence. While localized melanoma can be treated with excision, there are at present no similarly effective treatments for regional and distant disease, so survival rates are low. One problem is that melanoma is chemo-resistant, and most chemotherapy doses are limited by systemic toxicity. A method for delivering high-dose chemotherapy directly to tumors and draining lymph nodes could have the advantage of allowing much higher effective doses with reduced systemic exposure.

Methods: Human melanoma cell line A-2058 tumor cells were injected into athymic mice. After tumors grew to 50~100 mm³ mice were divided into five groups: (1) nontreated (2) intravenous (i.v.) cisplatin, (3) i.v. nano hyaluronan-conjugated cisplatin (HA-Pt), (4) subcutaneous (s.c.) peri-tumoral cisplatin, and (5) s.c. peri-tumoral HA-Pt. All treatment groups received 3 weekly doses of 10 mg/kg.

Results: Tumors grew progressively in all control, i.v. cisplatin, and s.c. cisplatin groups. Tumors showed a trend toward slower growth in the i.v. HA-Pt group, but all animals died or were euthanized per protocol within 3 weeks of treatment. Tumors showed shrinkage only in the subcutaneous peri-tumoral HA-cisplatin group; one of these mice appeared to be cured.

Conclusions: Peri-tumoral HA-cisplatin may be shown potential as a therapeutic option in treatment of certain types of melanoma.

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INTRODUCTION

Melanoma is a deadly skin cancer, killing more than 9,000 Americans in 2012. Incidence is rising rapidly, to the point where 1 in 50 Americans will develop melanoma.¹ Stage at diagnosis is the main determinant of survival. While 5-year survival for localized melanoma is 98%, involvement of regional lymph nodes drops survival to 62%, and in distant metastatic disease survival is only 15%.¹

The main reason is that very early disease can generally be successfully treated with simple excision. However, once it has spread to the lymph nodes and beyond, melanoma becomes very hard to treat and survival decreases accordingly. This applies to "locally advanced" melanoma with lymph node involvement as well as widespread metastatic disease. One important reason for this is that melanoma is notoriously resistant to chemotherapy. Conventional chemotherapy does not result in high levels of penetration into tumors or lymph nodes. Therefore efficacy is limited by systemic toxicity. It has long been a goal to increase relative penetration of chemotherapy into tumors and lymph nodes. Here we report the first use of a novel peri-tumor injectable chemotherapy compound in an in-vivo murine model for locally advanced melanoma. We seek to answer the question of whether increased peri-tumoral dose translates into a measurable in vivo response.

MATERIALS AND METHODS

All chemicals were obtained from commercial suppliers and used without further purification unless otherwise noted. Hyaluronan (HA; 35 kDa) was purchased from Lifecore Biomedical (Chaska, MN) as sodium hyaluronate, which was cultured and produced by a microbial fermentation process. Cisplatin (CDDP) was obtained from AK Scientific (Union, CA). All other chemicals and cell culture supplies were purchased from Sigma-Aldrich Co (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Distilled water was used in syntheses, cell culture (sterilized by autoclaving) and animal experiments (sterilized by autoclaving). Human melanoma cell line A-2058 was obtained from American Type Culture Collection (ATCC, MA) and cultured according to ATCC protocol.

Synthesis of Hyaluronan-Cisplatin Conjugates

HA-Cisplatin (HA-Pt) conjugate was prepared as previously described.² Briefly, HA (50 mg) and CDDP (40 mg) were dissolved in a total of 80 mL double distilled water (ddH₂O) and stirred in the dark for 96 hr at ambient temperature (~25°C). By the end of the reaction, the mixture was filtered through a 0.22- μ m nylon membrane filter (Fisher Scientific; Pittsburgh, PA), followed by dialysis (MWCO 10,000 Da; Pierce, IL) against ddH₂O for 24 hr in dark with 4 water changes. The crude HA-Pt conjugate was concentrated by evaporation under reduced pressure and then stored at room temperature in dark.

The CDDP substitution degree was determined by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent Technologies 7500a) using terbium as internal standard. High purity argon (>99.996%) was used as carrier gas. The calibration concentrations of platinum included 1, 10, 20, 40, and 50 ppb.

Induction of Human Melanoma Xenografts

Human melanoma cell line A-2058 was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) L-glutamine-alanine at 37°C humidified atmosphere containing 5% CO₂. A-2058 cells were trypsinized and suspended in cell culture grade phosphate buffered saline (1x, pH 7.4) at a concentration of 5x10⁷ cells/mL for tumor inoculation.

All experimental procedures were approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC). Female athymic nude mice (~25 g, Charles River Laboratories; Wilmington, MA) were anesthetized with 2% isoflurane in oxygen, and 100-μL of A-2058 cell suspension was subcutaneously injected in the distal aspect of left mouse thigh and upper hind limb using a 27-ga needle to establish the tumor xenografts. Tumor growth was monitored twice per week via bi-dimensional measurement with a digital caliper, and the tumor volume was calculated using the equation:

$$\text{Tumor volume (mm}^3\text{)} = 0.52 \times (\text{width})^2 \times (\text{length})$$

Treatment

Female Nu/Nu nude mice bearing melanoma tumors of roughly 50~100 mm³ were randomly divided into five groups, including a non-treated group (N= 7) and four treatment groups (N=6-8). Three doses of 10 mg/kg CDDP or HA-Pt (on cisplatin basis) were administered weekly via tail vein for i.v. CDDP (N=6) group and i.v. HA-Pt (N=7) group; or subcutaneously near the tumor for s.c. CDDP (N=6) and s.c. HA-Pt (N=8) group. Following the treatment, the primary tumor size was measured twice weekly, and animals were euthanized once the tumor volumes reached 3000 mm³ or in the presence of necrosis or ulceration unrelated to tumor growth, or if weight loss >20% occurred.

Statistical Analyses

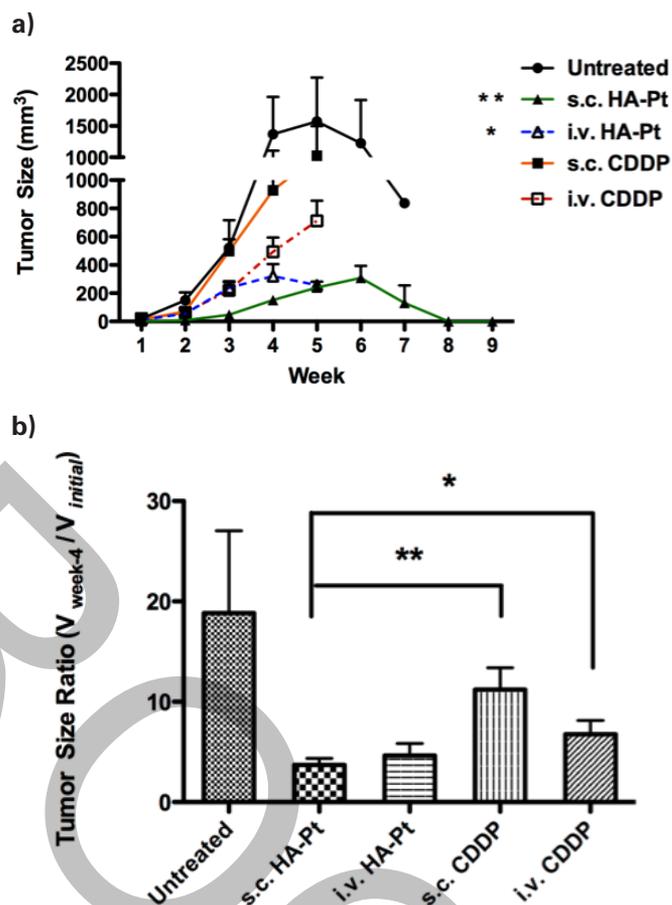
Tumor volumes and body weights for all five experimental groups were analyzed and expressed at mean ± standard deviation. Statistical analyses were conducted using unpaired t-test by GraphPad Prism 5 software with significance set at *P* < 0.05.

RESULTS AND DISCUSSION

Synthesis and Characterization of HA-Pt conjugate

Cisplatin was efficiently conjugated to HA with a conjugation degree of 20% wt through formation of ester linkages with

FIGURE 1. a) Tumor growth after treatment with s.c. HA-Pt, i.v. HA-Pt, s.c. CDDP, i.v. CDDP, and untreated control. CDDP and HA-Pt on equivalent platinum basis (10 mg/kg) were injected either by i.v. tail vein or s.c. around tumor, 6 ≤ N ≤ 8; **b)** Tumor Volume (*V*/*V*_{initial}) at week four after treatment, 5 ≤ N ≤ 8. (*: *P* < 0.05; **: *P* < 0.01)



polycarboxyl groups of the HA polymer.² The conjugate exhibited an in vitro release half-life of merely 10 hours.² A diameter of 7.55 ± 1.72 nm of individual particles or 25.2 ± 4.43 nm of large clusters formed by HA-Pt conjugate was revealed using Transmission Electron Microscopy.³

Therapeutic Efficacy

The maximum tolerated dose (MTD) of cisplatin has been determined at 10 mg/kg body weight in mice.⁴ The anti-tumor effect of HA-Pt and CDDP at MTD was evaluated by measuring tumor volume and survival times in nude mice bearing A2058 tumor xenografts. Skin tumors were observed in hind legs of nude mice within one week after tumor cell injection, and tumor volume typically reached ~100 mm³ in two weeks. Following treatment, tumor burden was monitored up to nine weeks. For mice receiving no anticancer treatment, average tumor size was 1,600 mm³ by week 5 (Figure 1A) and an average of 19-fold increase in tumor volume was observed from

FIGURE 2. Survival curve after treatment with untreated control, CDDP, or HA-Pt, respectively. CDDP and HA-Pt on equivalent platinum basis (10 mg/kg) were injected either by i.v. tail vein or s.c. around tumor, $6 \leq N \leq 8$. Of note, untreated and conventionally treated animals were euthanized either due to tumor size or severe necrosis at the injection site, while the s.c. HA-Pt animals were euthanized due to weight loss and Body Condition Score (BCS) < 2 . *BCS,⁵ is a useful tool for evaluating overall condition of the mouse.

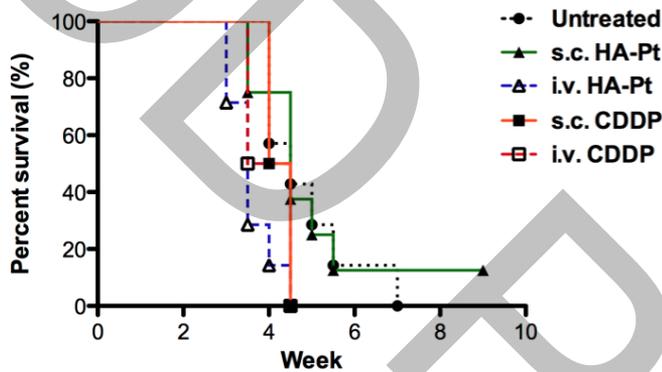
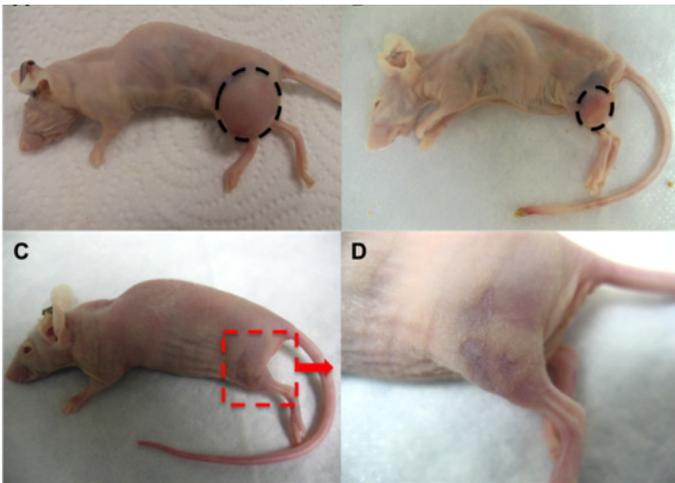


FIGURE 3. Nude mice bearing human A2058 melanoma tumor xenografts with untreated control **a**), BCS=2, tumor size = 2429 mm³, i.v. CDDP **b**) BCS =1, tumor size = 502 mm³, s.c. HA-Pt **c**) & **d**), BCS = 3, tumor size = 0 mm³. BCS of 1 or 2 indicates that the mouse is emaciated or under-conditioned, respectively. A mouse with BCS of 3, in comparison, is well conditioned.



week 1 to week 4 (Figure 1B). In contrast, tumors in animals treated with s.c. HA-Pt ($P < 0.01$) or i.v. HA-Pt ($P < 0.05$) developed at a significantly slower rate during the same period, with week 4 tumor volumes 83% smaller than those in the non-treatment group (Figure 1A). Animals that received s.c. CDDP and i.v. CDDP showed delayed tumor initial progression, but by week 4 their tumors had regrown by 12-fold and 7-fold vs week 1 sizes, respectively. In addition, 100% of the animals in two CDDP treated groups were euthanized within

5 weeks (Figure 2) either due to deteriorating body condition (Figure 3B) or necrosis of the injection site on the tail induced by the extravasation side effect of CDDP. By comparison, one mouse that was treated with s.c. HA-Pt showed complete tumor eradication and the mouse lived through this study (Figure 3D). Other s.c. HA-Pt mice were euthanized primarily due to weight loss, not tumor growth or ulceration.

DISCUSSION

Locally advanced cancer refers to cancers that have spread from where they started to nearby tissues or lymph nodes.⁶ Such cancers generally cannot be adequately treated by surgical resection alone. This report presents the results of a locally delivered chemotherapy treatment in a murine model for locally advanced melanoma.

Although treatment of melanoma has demonstrated incremental improvement over the last few decades, in 2011 the five-year death rate for stage 3 locally advanced melanoma with lymphatic metastases was still 37.6%.¹ Over 6000 people are diagnosed with stage 3 melanoma per year in the US.⁷ There is at present no chemotherapy approved for direct delivery into locally advanced melanoma. Current standard-of-care treatment relies on surgery, and, in certain cases, local radiation and/or immunomodulators such as interferon- α or interleukin-2. Chemotherapy is generally reserved for disease that has progressed to distant metastases. Even so, traditional chemotherapeutic agents have not shown prolonged responses in most patients.

While newer agents have shown promise, none has yet demonstrated sustained efficacy for most treated patients. For instance, the BRAF inhibitor vemurafenib has been recently demonstrated a four-month survival advantage in patients with unresectable melanoma.⁸ The immune modulator ipilimumab, an anti-CTLA4 monoclonal antibody, has been shown to promote antitumor immunity and improve survival rates in patients with metastatic melanoma.⁹ A metanalysis showed a six-month increase in overall survival time for ipilimumab compared to chemotherapy.¹⁰ In patients with stage III and stage IV unresectable melanoma, combining ipilimumab with a gp100 peptide vaccine showed a four-month survival advantage vs vaccine alone (10.1 months vs 6.4 months).⁹ Though not yet approved, numerous vaccines are currently being developed for the treatment of melanoma, including dendritic cell vaccines and herpes simplex virus oncolytic "vaccines".

Current immune treatments such as ipilimumab carry risks for significant side effects including hepatic and gastrointestinal toxicity, endocrine dysfunction, and permanent retinal damage. Ocular side effects include conjunctivitis, scleritis, uveitis,¹¹ decreased visual acuity, photophobia, and painful tearing,¹² as well as Graves ophthalmopathy.^{11,13}

Traditional chemotherapy is not typically used in locally advanced melanoma, but it can play a role in treating distant metastases. Cisplatin, also known as CDDP, is one such potential chemotherapy candidate. CDDP is a DNA-damaging chemotherapeutic agent that induces apoptosis in tumor cells. CDDP achieves higher concentrations in melanoma tissue than plasma. In a patient receiving IV CDDP for cutaneous melanoma metastases, platinum levels in tumor tissue were three times higher than plasma.¹⁴ Although widely used across a broad variety of cancers, CDDP has played only a limited role in melanoma treatment. CDDP has not shown great efficacy as melanoma monotherapy, likely due to dosing limitations.

CDDP has been studied in combination with other melanoma treatments including immunotherapy,¹⁵⁻¹⁷ gene therapy,¹⁸⁻²⁰ and other small-molecule anticancer agents.^{4,21,22} These studies have used both murine and human melanoma lines in mouse xenotransplantation models, and have demonstrated synergistic inhibitory effects resulting in significant retardation or even complete tumor growth inhibition. The enhanced cytostatic/cytotoxic effects might be attributed to coupling tumor cell apoptosis induced by CDDP with anti-proliferative, antiangiogenic or anti-cell cycle effects of other agents. The precise mechanisms underlying the synergies have not yet been fully elucidated.

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Of note, when augmented anti-tumor effects were found in combination immuno-chemo studies, they typically used intensive CDDP treatment schedules that would not likely be tolerated by human patients.¹⁸ Delivering chemotherapy directly to tumor and local lymph nodes could result in much more of the chemotherapy actually getting to where it is needed. This might be even more advantageous in treating locally advanced disease.

Here we present a new method for achieving very high doses of CDDP in locally advanced melanomas. We previously reported synthesis of an injectable hyaluronan-CDDP nanoconjugate (HA-Pt) using the FDA-approved biocompatible carrier hyaluronan (HA).²³ HA-Pt can be administered subcutaneously as a peri- or intra-tumoral injection. As such, it has the potential to enhance drug accumulation in the primary tumor and adjacent lymph nodes, while simultaneously reducing overall systemic exposure.^{2,3,23} Nodal penetration is important since local nodes are where most tumor metastases start. HA-Pt has previously demonstrated improved anti-tumor efficacy

in in vivo murine models of locally advanced head and neck squamous cell carcinoma²⁴ and breast cancer.²⁵ The present study provides the first experimental evidence that HA-Pt can inhibit human melanoma growth in mice.

HA-conjugation may be especially helpful because HA is a ligand for the tumor-associated surface receptor CD44. Numerous studies have shown that CD44 is expressed in both primary and metastatic melanoma.^{26,27} CD44 is the main hyaluronic acid surface receptor on melanoma cells, and CD44 expression increases during melanoma progression.²⁸ CD44 expression in melanoma cells has also been associated with worsening Clark's stage.²⁶ Although CD44-hyaluronic acid interaction has been reported to induce melanoma cell proliferation,²⁸ when the hyaluronan in question is conjugated to a poison it is would not appear likely to promote tumor survival. Furthermore, a recent report found that not only was CD44 expressed in all tumor stromal cells studied, but CD44 expression by tumor stromal precursors played vital roles in tumor migration, incorporation, and functionality.¹³ Therefore HA-Pt could potentially target tumor stroma as well as neoplastic cells.

The ability to shrink melanomas via direct injection of HA-Pt could be of clinical interest in several circumstances, including augmentation or even substitution for complete lymph node dissection (CLND) or radiation, preoperative shrinkage of very large primary tumors, palliative shrinkage of large or awkwardly located metastases, and synergistic combination with immunotherapy.

Radiation therapy has been used as adjuvant therapy following CLND, and as alternate therapy for affected regional lymph nodes. When used as adjuvant therapy after lymphadenectomy, only 10% of patients who received radiation developed regional recurrence after five years, as opposed to 40% of patients who did not receive radiation.²⁹ Another study demonstrated doubling of disease-free survival time when radiation therapy was used to treat regional lymph nodes.³⁰ Radiation has also been used in the treatment of skin metastases; however, treatments of over 6 Gy were needed to elicit response rates above 90%.³¹ Since cisplatin has been shown to sensitize cancer cells to ionizing radiation, targeting CDDP to nodes via HA-Pt could be useful.³² This might be especially helpful in patients who are not candidates for full-dose systemic CDDP or high-dose radiation.

Because complete lymph node dissection is associated with serious morbidities, including lymphedema, bleeding, infection, and deep vein thrombosis,³³ some patients are unable or unwilling to undergo CLND despite positive sentinel lymph node or other risk factors for nodal involvement. Such patients may benefit from such a treatment that can deliver a large dose to the local nodes with relatively low systemic exposure and a correspondingly high therapeutic index.

Shrinking very large melanomas prior to surgery could improve cosmesis and also improve cure rates especially in certain areas the body such as the face, hands or feet where wide margins are hard to achieve. Similarly, patients with large or obstructive metastases could potentially benefit from direct injection of HA-Pt for palliative tumor masses shrinkage. Doing so might provide benefit with fewer side effects than such conventional treatments as high-dose chemotherapy or repeated surgical debulking.

Mouse and human studies have explored combined use of platinum-based antineoplastic drugs (eg, cisplatin or carboplatin) and immunotherapy (eg, interleukin-2 or interferon-alpha 2a). One combination therapy demonstrated synergistic inhibition of advanced B16-F1 melanoma growth in syngeneic mice¹⁶ and another combination therapy study showed at least additive benefit with mostly moderate toxicity in two consecutive phase II trials with a total of 85 patients.¹⁷ In our murine melanoma study, local administration of HA-Pt showed improved efficacy vs standard i.v. CDDP. HA-Pt may therefore potentially be of benefit in sequential chemo/immunotherapy protocols.

Although further work is needed, these data provide preliminary support for the proposition that HA-Pt may potentially play a role in treating locally advanced melanoma and other similar conditions.

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DISCLOSURES

Drs. Cai, Aires, and Forrest are involved in NanoPharm dba HylaPharm, a company seeking to develop nano-hyaluronan anti-cancer technologies.

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AUTHOR CORRESPONDENCE

M. Laird Forrest PhD

E-mail:.....mforrest@ku.edu