Research Article

Abrogating Drug Resistance in Malignant Peripheral Nerve Sheath Tumors by Disrupting Hyaluronan-CD44 Interactions with Small Hyaluronan Oligosaccharides

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Abstract

Malignant peripheral nerve sheath tumors (MPNST) develop in ~10% of neurofibromatosis type-1 patients and are a major contributing factor to neurofibromatosis-1 patient mortality and morbidity. MPNSTs are multidrug resistant, and thus long-term patient survival rates are poor after standard doxorubicin or multiagent chemotherapies. We show that the hyaluronan receptor CD44 forms complexes with multidrug transporters, BCRP (ABCG2) and P-glycoprotein (ABCB1), in the plasma membrane of human MPNST cells. Small hyaluronan oligosaccharides antagonize hyaluronan-CD44–mediated processes and inhibit hyaluronan production. Treatment of MPNST cells with the hyaluronan oligomers causes disassembly of CD44-transporter complexes and induces internalization of CD44, BCRP, and P-glycoprotein. Consequently, the oligomers suppress drug transporter activity and increase sensitivity to doxorubicin treatment in culture. *In vivo*, systemic administration of hyaluronan oligomers inhibits growth of MPNST xenografts. Moreover, the oligomers and doxorubicin act synergistically *in vivo*, in that combined suboptimal doses induce tumor regression to a greater extent than the additive effects of each agent alone. These findings indicate that constitutive hyaluronan-CD44 interactions contribute to drug transporter localization and function at the plasma membrane, and that attenuating hyaluronan-CD44 interactions sensitizes MPNSTs to doxorubicin *in vitro* and *in vivo*. These results also show the potential efficacy of hyaluronan oligomers, which are nontoxic and nonimmunogenic, as an adjuvant for chemotherapy in MPNST patients. [Cancer Res 2009;69(12):4992–8]

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant, neuro-cutaneous disorder with an estimated incidence of 1:2,500 to 1:3,000 people worldwide (1, 2). The absence of neurofibromin in NF1 causes an array of clinical manifestations, including tumors such as gliomas, neurofibromas, plexiform neurofibromas, and malignant peripheral nerve sheath tumors (MPNST). MPNSTs arise in ~10% of patients with NF1 and are a major contributing factor to NF1 patient mortality and morbidity. MPNSTs are believed to arise either spontaneously or from preexisting neurofibromas, which are benign peripheral nerve sheath tumors composed primarily of Schwann cells and fibroblasts. In contrast, MPNSTs are rapidly growing, spindle-cell sarcomas that infiltrate surrounding tissues and frequently metastasize to the lungs, lymph nodes, and liver. Moreover, MPNSTs are characteristically resistant to therapy. Surgical resection, local radiotherapy, and doxorubicin or multiagent chemotherapy are mainstays of MPNST treatment, but long-term survival rates are poor (3, 4).

Cancer cells exhibit multiple mechanisms of resistance to chemotherapeutic agents, including enhanced drug efflux via the ATP-binding cassette (ABC) family transporters (5–8). Hyaluronan, a large extracellular glycosaminoglycan that is enriched in a variety of tumors, including MPNSTs (9, 10), has been shown to influence chemoresistance (11, 12). Interactions of hyaluronan with its major cell surface receptor, CD44, increase resistance to numerous drugs (13–18) and stimulate expression of the drug transporters, breast cancer resistance protein/ABCG2 (BCRP; ref. 19), and P-glycoprotein/ABCB1 (Pgp; ref. 14). Moreover, CD44 interacts with Pgp in tumor cell membranes (20, 21).

Disruption of constitutive hyaluronan-CD44 interactions suppresses multidrug resistance and malignant cell properties at several different levels (e.g., through inactivation of receptor tyrosine kinases, cell survival signaling pathways, and drug transporters; ref. 12). In our previous studies, we have used several antagonists of hyaluronan-CD44 interactions to probe their function in these activities. For example, small hyaluronan oligosaccharides (o-HA) compete with polymeric hyaluronan for binding to receptors such as CD44 (22). Whereas polymeric hyaluronan binds to CD44 multivalently and with high affinity, o-HA containing 6 to 18 sugar residues bind monovalently and with low affinity (23). Prolonged treatment of cancer cells with these o-HA inhibits drug transporter expression and sensitizes the cells to drugs, including doxorubicin, Taxol, vincristine, and methotrexate (13, 14, 18, 19). o-HA also inhibit hyaluronan production (24), presumably through interference with interactions within the hyaluronan synthase apparatus, which is also located at the plasma membrane (25).

In the present study, we examine interactions of CD44 with BCRP and Pgp in the membrane of MPNST cells and the influence of o-HA on transporter localization and function in these cells. We find that CD44 associates with BCRP and Pgp at the plasma membrane, and that treatment of MPNST cells with o-HA induces complex disassembly and internalization of CD44, BCRP, and Pgp from the membrane into the cytoplasm. Consequently, o-HA treatment decreases doxorubicin resistance, inhibits efflux of the Pgp substrate FURA 2-AM, and suppresses hyaluronan secretion. Moreover, using an MPNST xenograft model in nude mice, we show that o-HA induce tumor regression and sensitize the tumors to

doxorubicin treatment in a synergistic manner. Together, these data indicate that constitutive, cell-autonomous interactions between hyaluronan and CD44 contribute to ABC-family transporter localization and function *in vitro* and *in vivo*, showing the potential efficacy of o-HA as adjuvant therapy for drug-resistant MPNSTs.

Materials and Methods

Cells and reagents. ST88-14 human MPNST cells were obtained from Dr. Larry Sherman (Oregon Health and Science University, Portland, OR). Fetal bovine serum (FBS) was purchased from Atlas Biologicals. DMEM and Ham's F12 were purchased from Sigma. Growth factor–reduced Matrigel matrix was purchased from BD Biosciences. The following antibodies were obtained for these studies: BCRP clone BXP-21 (Kamiya Biomedical Company), HCAM (DF1485; Santa Cruz Biotechnology), FITC-tagged CD44 (Cedarlane Laboratories), Pgp (Calbiochem), β-actin (Sigma), goat anti-mouse horseradish peroxidase (HRP), and goat anti-rabbit HRP (Chemicon). Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 secondary antibodies and Alexa Fluor 647 phalloidin were purchased from Invitrogen. DRAQ5 nuclear stain was obtained from Biostatus Limited. Western blotting detection reagent (ECL) was purchased from Pierce. Cell proliferation assay reagent, WST-1, was obtained from Takara Bio, Inc. FITC-labeled o-HA and hyaluronan polymer were generously donated by Anika Therapeutics. Unlabeled hyaluronan oligosaccharides used in this study were a mixed fraction of average molecular weight $\sim$2.5 $\times$ 10^3, composed of 2 to 10 disaccharide units, that were fractionated from testicular hyaluronidase (Sigma, type 1-S) digests of hyaluronan polymer (Sigma, sodium salt); fractionation was by trichloroacetic acid precipitation followed by serial dialysis, using membranes of Amicon Ultra Ultracel 5,000 MWCO (Millipore) and Spectra/Por Membrane 1,000 MWCO (Spectrum Laboratories). All other chemicals were of reagent grade or higher.

Cell culture. ST88-14 MPNST cells were cultured in DMEM/Ham's F12 with 2.38 g/L HEPES, 2 g/L sodium bicarbonate, and 10% FBS, pH 7.4, and maintained at 37°C in a humidified 5% CO_2-95% air incubator. For preparation of lysates, cells were seeded in 10-cm dishes 48 h before treatment with o-HA. For preparation of confocal microscopy slides, cells were seeded at low density in eight-well multichamber culture slides (BD Biosciences) 48 h before o-HA treatment.

Hyaluronan, WST, and FURA 2-AM assays. Hyaluronan levels were assayed in conditioned medium by an ELISA-like assay (26). WST assay was done according to the manufacturer's protocol. FURA 2-AM efflux was measured as described (27): MPNST cells were seeded in 96-well plates and incubated for 48 h before the experiment to a final density of $\sim$70% confluence. Cell were treated –/+ 100 µg/mL o-HA in feed medium containing 2.5 µmol/L FURA 2-AM. Following a 2-h incubation, plates were read (excitation, 340 nm; emission, 500 nm) in an FLx800 Microplate Fluorescence Reader (Biotek Instruments, Inc.) from the top and bottom to determine FURA 2-AM levels in the conditioned medium and cell layer, respectively.

Immunoprecipitation and immunoblot analysis. Following a 1-h incubation with or without o-HA treatment, whole-cell lysates were prepared for immunoprecipitation or direct immunoblotting as previously described (28), and aliquots containing 50 or 500 µg of protein were solubilized in SDS sample buffer for immunoblotting or used in immunoprecipitation, respectively. Immunoprecipitation was carried out with the Catch and Release immunoprecipitation kit according to the manufacturer's specifications (Upstate Biotechnology) followed by immunoblot analysis as previously described (28).

Confocal microscopy. Cells were plated, fixed, permeabilized, blocked for nonspecific binding, incubated with the indicated antibodies, and washed as previously described (28). Cells were then incubated with Alexa Fluor secondary antibodies and Draq5 nuclear stain or Alexa Fluor phalloidin for 2 h. Slides were processed as described (28) and viewed on a Leica Total Confocal System, Spectral Prism 2, Acoustic Optical Beam Splitter (TCS SP2 AOBS) in the Josh Spruill Molecular Morphology and Imaging Center in the Department of Cell Biology and Anatomy. Quantitative pixel analysis was done with Canvas 8 software.

MPNST xenografts. Following trypsinization and a PBS wash, 250,000 MPNST cells suspended in a 1:1 ratio of PBS to Matrigel (0.5 mL) were injected s.c. into the flank of congenitally athymic nude mice (20–25 g FOXn1nu strain) with a 23-gauge needle via a 0.5-cc syringe. Tumor growth was measured in two dimensions with digital calipers every 48 h, and treatment was begun when tumors exceeded 40 mm^2. I.p. treatments in a constant volume of 200 µL PBS buffer were delivered every 48 h via a 28-gauge needle and 0.5-cc syringe for a course of 16 d.

Results

Suppression of drug resistance in MPNST cells by hyaluronan oligomers. Complementary to our previous findings with other types of cancer cells (13, 14, 19), we show here that o-HA decrease resistance to doxorubicin in MPNST cells (Fig. 1). One mechanism whereby hyaluronan might influence drug resistance is through effects on multidrug transporter activity. Therefore, we measured the effect of o-HA on efflux of FURA 2-AM, a fluorescent substrate for Pgp. In cells expressing ABC transporters, intact FURA 2-AM is rapidly transported out of the cell, whereas it is cleaved to FURA 2 and accumulates in the cytosol in the absence of ABC transporters (27, 29). Thus, transporter activity is inversely proportional to accumulation of cytosolic FURA 2 fluorescence. We found that o-HA treatment caused an increase in intracellular fluorescence and a reduction of fluorescence in the medium of cultured MPNST cells (Fig. 2), indicating that the oligomers inhibit Pgp transporter activity.

As stated above, it is known that o-HA compete for interaction between polymeric hyaluronan and its receptors (22, 23). However, recent results indicate that o-HA also inhibit hyaluronan production or secretion (24) and that hyaluronan may be secreted through multidrug transporters (30). Therefore, we examined the effect of o-HA on the level of hyaluronan secreted by MPNST cells. The o-HA reduced secreted hyaluronan in a dose-dependent fashion, with almost complete inhibition at 10 to 100 µg/mL (Fig. 3), amounts found previously to inhibit drug resistance and signaling events downstream of hyaluronan-CD44 interactions (13, 14, 19).
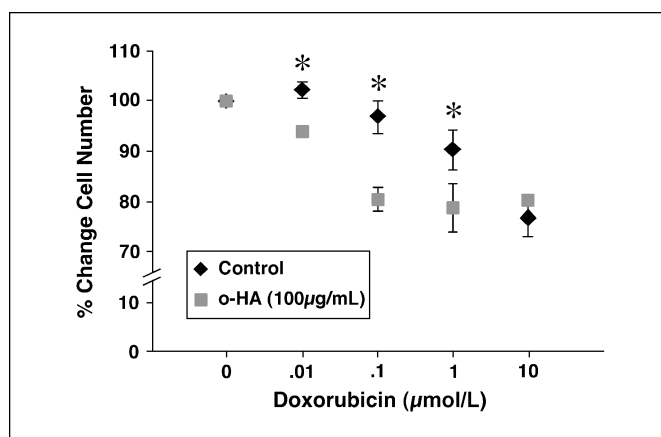
Figure 1. Hyaluronan oligomer treatment decreases resistance to doxorubicin. MPNST cells were treated with various doses of doxorubicin –/+ o-HA (100 µg/mL) for 48 h, after which cell number was determined with the WST colorimetric cell proliferation reagent. *Points,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$, significant differences between o-HA–treated and untreated cells at 0.01 to 1.0 µmol/L doxorubicin (Student's *t* test). Representative results of three or more independent experiments.
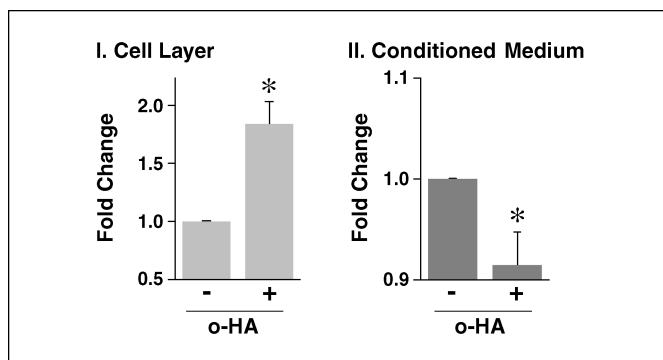
Figure 2. Hyaluronan oligomer treatment decreases FURA 2-AM export. MPNST cells were treated –/+ o-HA (100 μg/mL) in feed medium containing 2.5 μmol/L FURA 2-AM. Following a 2-h incubation, cell layer (*I*) and conditioned medium (*II*) were analyzed for fluorescence as described in Materials and Methods. *Columns,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$, significant differences between o-HA–treated and untreated cells (Student's *t* test). Representative results of three or more independent experiments.

These results led us to examine the effects of o-HA on ABC transporters in MPNST cells more closely.

CD44-multidrug transporter interactions in MPNST cells. We chose to study the effects of o-HA on CD44 interactions with two transporters (i.e., BCRP and Pgp) because we showed that prolonged (24 hours) o-HA treatment decreases their expression (13, 14, 19) and because Pgp has been shown to interact with CD44 by other investigators (20, 21). In addition, chemotherapeutic drugs currently used in treatment of MPNSTs are substrates of these transporters.

First, we analyzed lysates of MPNST cells for the presence of CD44, BCRP, and Pgp. As expected from previous publications (10, 31), MPNST cells express high levels of standard CD44 (∼85 kDa) and lower but significant levels of variant CD44 isoforms (>105 kDa; Fig. 4*A*). The MPNST cells also express both BCRP and Pgp (Fig. 4*A*). The levels of expression of CD44, BCRP, and Pgp are not significantly affected by treatment with o-HA for 1 h (Fig. 4*A*).

We then sought evidence for interactions between CD44 and the transporters by performing immunoprecipitations from lysates of the MPNST cells. Using antibody against CD44 for immunoprecipitation and subsequent immunoblotting with antibodies against BCRP and Pgp, we found both transporters in the CD44 immunoprecipitates (Fig. 4*B*). We also performed immunoprecipitations with antibodies against BCRP (Fig. 4*C*) and Pgp (Fig. 4*D*), both of which pulled down CD44. As previous work in our lab has shown that signaling complexes containing CD44 and receptor tyrosine kinases (32, 33) or monocarboxylate transporters (28) are dissociated by treatment of cells with antagonists of hyaluronan-CD44 interaction, we determined whether o-HA also affect interactions between CD44 and BCRP or Pgp. Treatment of MPNST cells with o-HA for 1 h decreased the amounts of BCRP and Pgp that appeared in the complexes immunoprecipitated with CD44 antibody (Fig. 4*B*). Complementary immunoprecipitations with BCRP (Fig. 4*C*) or Pgp (Fig. 4*D*) also showed a modest but consistent decrease in the amount of CD44 complexed with these transporters after treatment of the cells with o-HA for 1 h. As noted previously (28), we have found that the "pan"-CD44 antibody used for immunoprecipitation is somewhat inefficient and variable in its ability to pull down variant forms of CD44, even though it recognizes them clearly in immunoblots. This may explain some of the variability observed in these immunoprecipitations, especially because our

previous study showed that monocarboxylate transporters preferentially associate with variant forms of CD44 (28). In addition, the greater effects of o-HA seen in the CD44 immunoprecipitations compared with the BCRP or Pgp immunoprecipitations are most likely due to different antibody sensitivities, implying that disassembly of the complexes is not complete under the conditions used. This conclusion is supported by the confocal microscopy results presented below.

Internalization of CD44 and multidrug transporters by treatment of MPNST cells with hyaluronan oligomers. To further explore interactions between CD44 and BCRP or Pgp, we examined the localization of each protein by confocal microscopy. In Fig. 5, we show that CD44 is colocalized with BCRP (Fig. 5*A, a*) and Pgp (Fig. 5*B, a*) at the plasma membrane of MPNST cells (colocalization: yellow signal). For comparison, image *e* of Fig. 5*A* and *B* shows BCRP or Pgp alone, and image *c* of Fig. 5*A* and *B* shows CD44 alone at the cell membrane. These results, together with those in the previous section, indicate that CD44 interacts directly or indirectly with BCRP and Pgp at the plasma membrane of MPNST cells.

The immunoprecipitation experiments suggested that treatment with o-HA may decrease interactions between CD44 and drug transporters. Thus, we examined the influence of o-HA on the localization of CD44, BCRP, and Pgp. After treatment of MPNST cells with o-HA for 1 h, followed by fixation and processing for confocal microscopy, we found that each of these three plasma membrane components became localized in the cytoplasm and that internalization was accompanied by a significant but not complete loss of colocalization of CD44 and the ABC transporters in the cell membrane (Fig. 5*A* and *B*—*b, d*, and *f*). Quantitative pixel analyses revealed ∼3-fold more colocalization of CD44 and BCRP over the whole cell and 60-fold more colocalization in the plasma membrane of untreated cells when compared with o-HA–treated cells. Similarly, we found 25-fold more colocalization of CD44 and Pgp over the whole cell and 400-fold more colocalization in the plasma membrane of untreated cells than in o-HA–treated cells.
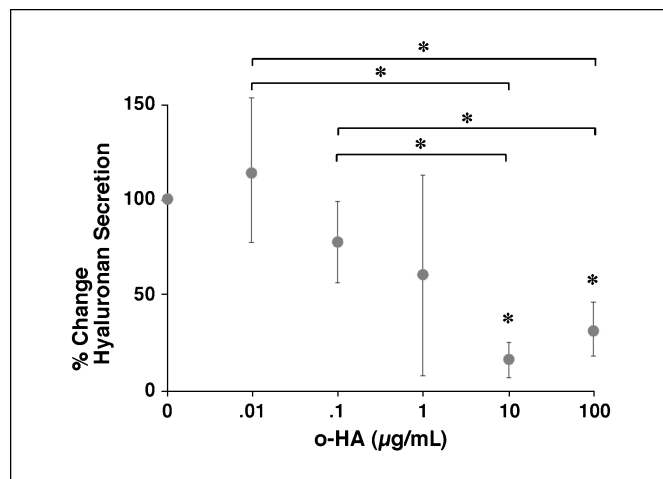
Figure 3. Hyaluronan oligomer treatment decreases secreted hyaluronan. MPNST cells were treated for 24 h with various doses of o-HA. Conditioned medium was collected, and hyaluronan quantified by an ELISA-like assay. Results were normalized to cell number. *Points,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$, significant differences between o-HA–treated and untreated cells at 10 and 100 μg/mL o-HA (one-way ANOVA with Bonferroni comparison between points). Significant differences (*, $P < 0.05$) were also observed between various o-HA concentrations, as indicated by brackets. Representative results of three or more independent experiments.

To confirm that internalization of CD44 in response to treatment with o-HA was an active cellular process, we treated live cells with Alexa Fluor 488–conjugated CD44 antibody in the absence or presence of o-HA for an hour before fixation. Following fixation, we also stained actin filaments with phalloidin. As in the previous experiments wherein CD44 antibody staining was done after fixation (Fig. 5*A* and *B*), we observed that CD44 became localized within the cytoplasm following oligomer treatment (Fig. 5*C, b* and *d* versus *a* and *c*). Image *a* of Fig. 5*C* also shows colocalization of CD44 with phalloidin-stained cortical actin.

As a control for nonspecific charge effects of o-HA, we compared treatment of living cells with FITC-tagged o-HA versus FITC-tagged hyaluronan polymer, followed by fixation and staining for CD44 and the nucleus (Fig. 5*D*). FITC-tagged o-HA treatment for 1 h induced internalization of CD44 (Fig. 5*D, h*), whereas treatment with FITC-tagged hyaluronan polymer for 1 h gave results that were indistinguishable from the FITC control (Fig. 5*D, i* versus *g*). Interestingly, FITC-tagged o-HA was internalized to a perinuclear region of the cytoplasm (Fig. 5*D, e*) in association with CD44 (Fig. 5*D, b* and *h*; note perinuclear yellow signal in *b*). In contrast, FITC-tagged hyaluronan polymer remained primarily at the cell membrane (Fig. 5*D, f*) in association with CD44 (Fig. 5*D, c* and *i*; note membrane-associated yellow signal in *c*).

Synergistic induction of MPNST xenograft regression by systemic administration of hyaluronan oligomers and doxorubicin. To explore the ability of o-HA to affect MPNST growth *in vivo*, we established s.c. MPNST xenografts in the flanks of nude mice. Initial experiments revealed that a single intratumoral (i.t.) injection of o-HA, at doses as low as 0.5 mg/kg, suppressed tumor growth (data now shown). Subsequently, using i.p. injections, we compared the effects on MPNST growth of various concentrations of o-HA alone, doxorubicin alone, and o-HA in combination with doxorubicin, delivered every 48 hours for 2 weeks. In Fig. 6*A*, we show that a dose of 0.5 mg/kg of o-HA suppresses tumor growth and that 5 mg/kg causes tumor regression. However, doses higher than 5 mg/kg [i.e., 50 mg/kg (not shown) and 250 mg/kg (Fig. 6*A*)] did not seem to increase tumor regression significantly beyond that obtained with 5 mg/kg. In Fig. 6*B*, we show that a dose of 1 mg/kg doxorubicin results in a small degree of tumor regression, and that increasing the dose to 5 mg/kg has little additional effect. Finally, in Fig. 6*C*, we show that combined suboptimal doses of o-HA (0.5 mg/kg) and doxorubicin (1 mg/kg) result in significant tumor regression, exceeding the additive effects of each agent alone or the individual effects of a 10-fold greater dose of o-HA alone or 5-fold greater dose of doxorubicin alone. These results, together with our *in vitro* data, strongly point toward a synergistic effect wherein o-HA sensitizes MPNST cells to doxorubicin by decreasing drug efflux.

Discussion

In this study, we have found that CD44 associates with the drug transporters, BCRP and Pgp, in the plasma membrane of MPNST cells. We also showed that an antagonist of hyaluronan-CD44 interactions (i.e., o-HA) induces disassembly of these complexes, inhibits transporter function, and decreases resistance to doxorubicin in MPNST cells *in vitro* and *in vivo*.

Previous studies by other laboratories have shown interrelationships between CD44 and Pgp. Using confocal microscopic colocalization and fluorescence resonance energy transfer studies in NIH-3T3 cells, Pgp and CD44 were found to interact within plasma membrane lipid microdomains (34). In addition, coimmunoprecipitation of Pgp and CD44 has been shown with carcinoma and melanoma cells (20, 21). In this study, we have shown by coimmunoprecipitations and confocal microscopy that both BCRP and Pgp are in close association with CD44 in the plasma membrane of MPNST cells. We have previously shown that complexes containing CD44 in association with ERBB2 and other signaling moieties are present in lipid microdomains within the plasma membrane of carcinoma cells, and that stimulation of hyaluronan production induces assembly of these complexes (32). Other investigators have documented analogous relationships of hyaluronan-CD44 interaction with receptor tyrosine kinases (35–38) and the Na$^+$-H$^+$ exchanger 1 (39). We have recently shown a similar relationship with the monocarboxylate (lactate) transporters, MCT1 and MCT4 (28). These findings indicate that CD44 interacts with a wide range of plasma membrane components and facilitates their functions.

In the current study, we have shown that o-HA inhibit cellular efflux of FURA 2-AM, a substrate for the ABC-transporter, Pgp, and induce internalization of BCRP and Pgp into the cytoplasm. In parallel, the o-HA inhibit hyaluronan production, suggesting that o-HA
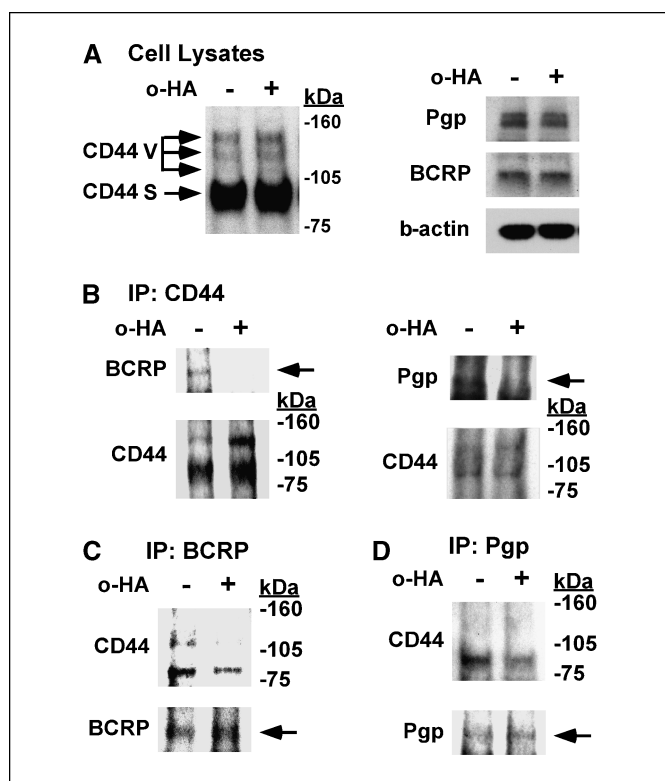
Figure 4. CD44 forms complexes with BCRP and Pgp. *A*, MPNST cells were treated for 1 h –/+ 100 μg/mL o-HA. Western blot analysis of whole-cell lysates (50 μg/lane) reveals strong expression of standard CD44 (*S*; ~85 kDa) and lower levels of splice variants (*V*; >105 kDa). The MPNST cells express both BCRP and Pgp. Expression of these proteins was not affected significantly by o-HA treatment. *B* to *D*, immunoprecipitations (*IP*) were carried out with antibodies against CD44 (*B*), BCRP (*C*), or Pgp (*D*) from lysates of MPNST cells treated –/+ o-HA. Western blot analysis of BCRP, Pgp, and CD44 was done, as indicated (*arrows,* positions of BCRP and Pgp bands). BCRP and Pgp were observed in the CD44 immunoprecipitate; CD44 was observed in both BCRP and Pgp immunoprecipitates. Treatment with o-HA caused a consistent decrease in the coimmunoprecipitated components. Representative blots of three or more independent experiments.
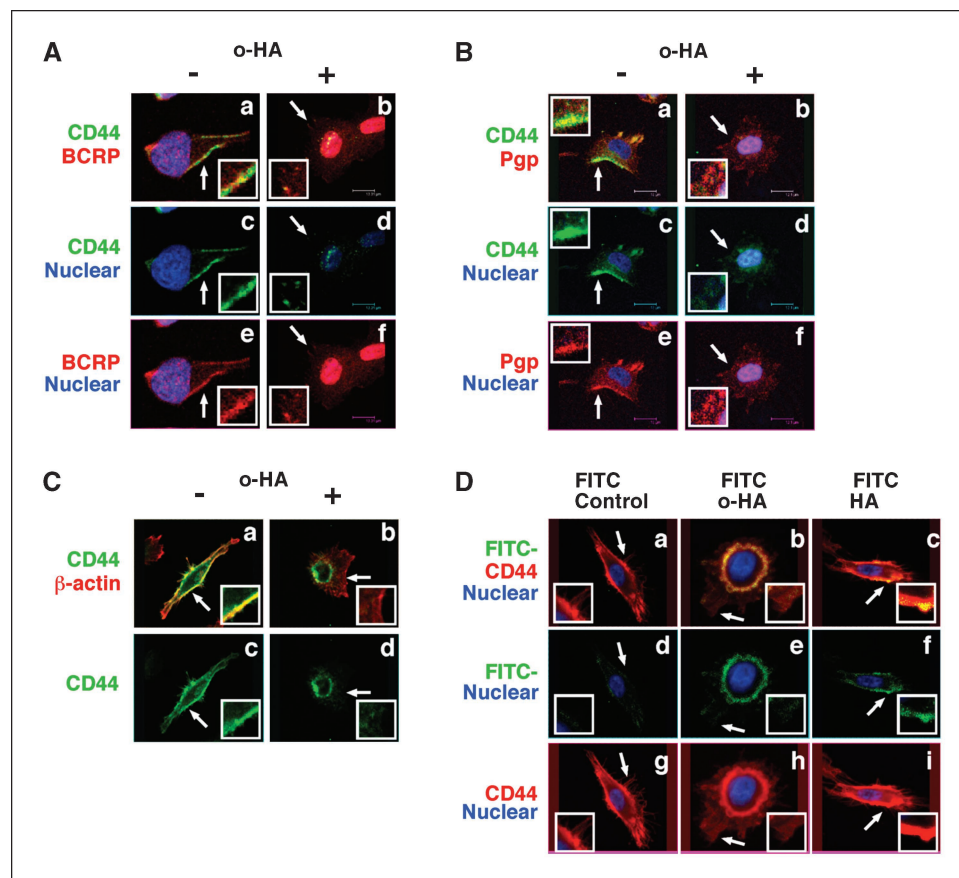
Figure 5. Hyaluronan oligomer treatment induces internalization of CD44, BCRP, and Pgp. *A* and *B,* MPNST cells were treated for 1 h +/− o-HA (100 μg/mL). Cells were stained with antibodies against CD44 (*green*) and BCRP (*red*; *A*) or Pgp (*red*; *B*) as well as Draq5 nuclear stain (*blue*), then visualized by confocal microscopy at a z-plane near to the center of the cell. *Arrows,* plasma membrane and the adjacent cytoplasm (shown at higher magnification in the insets). Colocalization of CD44 with BCRP and Pgp (*yellow signal*) is seen in the plasma membrane of untreated cells (image *a* of *A* and *B*), but not in the membrane of oligomer-treated cells (*b*). Selecting only for yellow pixels, quantitative analysis showed that colocalization of CD44 with BCRP (*A*) was 60-fold greater, and with Pgp (*B*) was 400-fold greater, in the plasma membrane of control cells than o-HA–treated cells. *C,* live MPNST cells were treated +/− o-HA (1 h) in the presence of Alexa Fluor 488–conjugated CD44 antibody (*green*), then processed for phalloidin staining of β-actin (*red*). *a,* double staining for CD44 and actin (*yellow,* colocalization); *c,* CD44 only. Note internalization of CD44 after treatment with o-HA (*b* and *d*). *D,* live MPNST cells were treated for 1 h with Alexa Fluor 488 (*FITC control*), Alexa Fluor 488–conjugated o-HA (*FITC o-HA*), or Alexa Fluor 488–conjugated hyaluronan polymer (*FITC HA*; *green*), and processed for staining with Alexa Fluor 555–conjugated CD44 (*red*) and Draq5 nuclear stain (*blue*). Note the plasma membrane localization of CD44 in the control Alexa Fluor 488–treated cells (*a* and *g*) and the colocalization of CD44 and hyaluronan in the plasma membrane of Alexa Fluor 488–conjugated hyaluronan polymer-treated cells (*c, f,* and *i*). In contrast, treatment with Alexa Fluor 488–conjugated o-HA resulted in internalization of both CD44 (*h*) and Alexa Fluor 488–conjugated o-HA (*e*) and their colocalization (*b*) in the cytoplasm. *Arrows,* plasma membrane and the adjacent cytoplasm (shown at higher magnification in the insets). Representative of three or more independent experiments.

may act, at least in part, by interfering with plasma membrane–associated hyaluronan synthase activity and/or reducing hyaluronan extrusion. Of interest in this regard is the observation that hyaluronan might be secreted through multidrug transporters in vertebrate cells (30, 40). These findings support a close relationship between hyaluronan production or secretion and the activity of ABC family drug transporters. Moreover, it is possible that inhibition of hyaluronan synthesis would decrease the level of CD44 occupancy and thus induce or promote the events described herein. In previous studies, we showed that both o-HA and CD44 siRNA inhibit drug resistance (14). However, whereas o-HA reproducibly inhibit hyaluronan production, CD44 siRNA does not (24, 33), suggesting that the effect on hyaluronan production is not necessary for inhibition of drug resistance. On the other hand, we find that o-HA remain associated with CD44 during its internalization (Fig. 5D). Thus, as previously proposed (11), o-HA most likely act by displacement of endogenous hyaluronan polymer from CD44, replacing the endogenous multivalent hyaluronan polymer-CD44 interaction with a monovalent interaction between o-HA and CD44. Nevertheless, it

is still possible that inhibition of synthesis amplifies the effects of the o-HA, and this possibility is being investigated.

Multivalent interactions between hyaluronan and CD44 may be necessary for stabilization of transporter interactions within the plasma membrane, and disruption of these interactions may then lead to endocytosis. In this regard, we previously found that inhibition of endogenous hyaluronan-receptor interactions, using o-HA, soluble hyaluronan-binding proteins, or siRNA against CD44, results in disassembly and inactivation of receptor tyrosine kinase-CD44 complexes (32, 33). Recent work shows that o-HA also cause rapid internalization of the receptor tyrosine kinases, ERBB2 and epidermal growth factor receptor (EGFR).[4] In similar fashion, we have shown that o-HA induce disassembly of CD44-lactate transporter complexes and internalization of both CD44 and the transporters (28). It is possible that o-HA prevent the initial assembly

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[4] Unpublished data.

of these complexes, rather than inducing disassembly and internalization, and that the loss of transporters and other signaling components from the cell surface is due to turnover. However, this alternative seems unlikely because the effects of the oligomers on internalization and function of drug transporters in the present study take place within an hour; similar results were obtained with lactate transporters (28). Current work is directed toward determining the fate of the transporters after treatment of the cells with o-HA.

These findings imply that hyaluronan-CD44 interactions stabilize many plasma membrane complexes and that a common mechanism may be involved. It is unclear as yet whether stabilization of a particular complex is due to unique interactions with a subpopulation of variant CD44, effects on organization of membrane compartments such as lipid rafts, or influences of various components within a hyaluronan-dependent pericellular matrix. Nevertheless, our results herein indicate that CD44 resides in close molecular vicinity to BCRP and Pgp, and that constitutive hyaluronan-CD44 interaction stabilizes these transporters and modulates their function in the plasma membrane.

Finally, by establishing a human MPNST xenograft model in nude mice, we have been able to study the effects of o-HA on tumor growth and drug resistance *in vivo*. Preliminary data indicated that i.t. injection of o-HA results in significant inhibition of growth, as found previously for several tumor types using i.t. or local Alzet pump administration (19, 41, 42). However, we found in this study that systemic delivery of o-HA, through i.p. injection, has a similar

Figure 6. Hyaluronan oligomer treatment inhibits MPNST tumor growth and acts synergistically with doxorubicin. *A* to *C*, s.c. MPNST xenografts were established in the flank of nude mice and grown to an area of 40 mm². I.p. injections of various concentrations of o-HA (*A*), doxorubicin (*B*), or a combination of suboptimal doses of both o-HA and doxorubicin (*C*) were delivered every 48 h for 2 wk after growth to 40 mm². Data are shown as fold changes after the beginning of treatment. The combination of suboptimal doses of o-HA and doxorubicin (*C*) results in greater inhibition of tumor growth than either component individually or their additive effects. Scatter plots compare the effects of o-HA, doxorubicin, and o-HA plus doxorubicin at 48-h increments over the course of 2 wk. Bar graphs compare the effects of these treatments at days 2 and 14. *Bars,* SD in tumor growth in four or more animals. *, $P < 0.05$, significant differences between controls and treated animals at 2 and 14 d (one-way ANOVA with Bonferroni comparison of significant difference between points). Significant differences (*, $P < 0.05$) were also observed between various treatments, as indicated by brackets.

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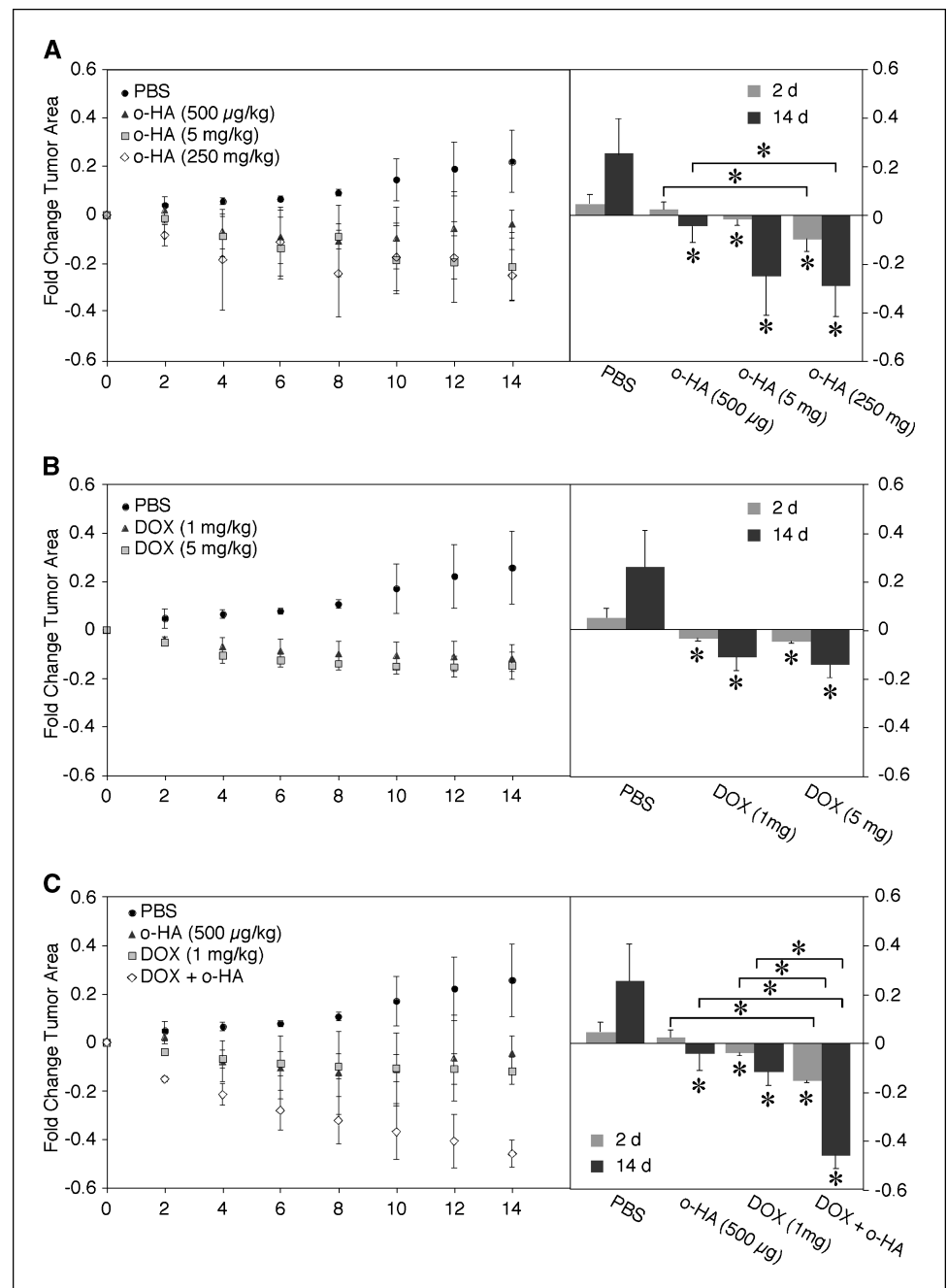
effect. Indeed, repeated doses of 500 µg/kg resulted in growth suppression, and doses of 5 mg/kg or more resulted in tumor regression over a 2-week period. Importantly, systemic injection of suboptimal doses of both o-HA (500 µg/kg) and doxorubicin (1 mg/kg) caused MPNST tumor regression to a degree greater than the additive effects of each reagent alone, and indicated a synergistic effect of the two agents.

In summary, our findings support a model in which constitutive hyaluronan-CD44 interaction stabilizes transporter interactions and activity in the plasma membrane, resulting in enhanced multidrug resistance. Our preclinical study with MPNST xenografts indicates that treatment with o-HA, which are nontoxic and nonimmunogenic, may be a novel and effective adjuvant to chemotherapy in MPNST patients.

Disclosure of Potential Conflicts of Interest

B.P. Toole: Inventor on a patent related to the article. The other authors disclosed no potential conflicts of interest.

Acknowledgments

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