Targeting N-cadherin Increases Vascular Permeability and Differentially Activates AKT in Melanoma

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Objective: We investigate the mechanism through which N-cadherin disruption alters the effectiveness of regional chemotherapy for locally advanced melanoma.

Background: N-cadherin antagonism during regional chemotherapy has demonstrated variable treatment effects.

Methods: Isolated limb infusion (ILI) with melphalan (LPAM) or temozolomide (TMZ) was performed on rats bearing melanoma xenografts after systemic administration of the N-cadherin antagonist, ADH-1, or saline. Permeability studies were performed using Evans blue dye as the infusate, and interstitial fluid pressure was measured. Immunohistochemistry of LPAM-DNA adducts and damage was performed as surrogates for LPAM and TMZ delivery. Tumor signaling was studied by Western blotting and reverse-phase protein array analysis.

Results: Systemic ADH-1 was associated with increased growth and activation of the PI3K (phosphatidylinositol-3-kinase)-AKT pathway in A375 but not DM443 xenografts. ADH-1 in combination with LPAM ILI improved antitumor responses compared with LPAM alone in both cell lines. Combination of ADH-1 with TMZ ILI did not improve tumor response in A375 tumors. ADH-1 increased vascular permeability without affecting tumor interstitial fluid pressure, leading to increased delivery of LPAM but not TMZ.

Conclusions: ADH-1 improved responses to regional LPAM but had variable effects on tumors regionally treated with TMZ. N-cadherin-targeting agents may lead to differential effects on the AKT signaling axis that can augment growth of some tumors. The vascular targeting actions of N-cadherin antagonism may not augment some regionally delivered alkylating agents, leading to a net increase in tumor size with this type of combination treatment strategy.

Keywords: AKT, drug delivery and N-cadherin, isolated limb infusion, melanoma, vascular permeability

Melanoma is the most aggressive form of skin cancer and ranks as the sixth most common cancer in the United States.1,2 Although early-stage melanoma can frequently be cured by surgical excision, patients with metastatic melanoma generally have a poor prognosis due to its aggressive tumor biology and chemoresistant nature.3 After appropriate initial therapy, as many as 10% of patients with primary extremity lesions will develop recurrence in the same extremity in the form of in-transit metastases.4,5 For these patients, the chemoresistant nature of metastatic melanoma can be partially overcome by infusing extremely high doses of cytotoxic agents through a surgically isolated extremity in the form of isolated limb infusion (ILI) or isolated limb perfusion.6 Using these techniques, we have reported overall response rates for ILI and isolated limb perfusion of 64% and 79%, respectively.7 Despite high overall response rates, most patients will eventually develop recurrence, supporting the role for novel research aimed at improving durable responses and minimizing toxicity.8

Combining regional chemotherapy with targeted therapies directed against pathways associated with melanoma remains a promising strategy for improving both the efficacy of the chemotherapeutic agent and the durability of the antitumor response. During the malignant transformation of normal melanocytes, there is a switch in cadherin expression. E-cadherin (generally expressed in normal epithelial cells) is downregulated, and N-cadherin (overexpressed in several malignancies) is upregulated. This switch alters intracellular signaling pathways, resulting in increased proliferation, migration, and survival.8-12 ADH-1 is a cyclic pentapeptide that disrupts N-cadherin interactions; it has been shown to inhibit cell growth and tumor progression both in vitro and in vivo.11,12 On the basis of strong preclinical evidence supporting synergism of systemic ADH-1 and regionally infused melphalan (LPAM),13 phase I and phase II clinical trials have been conducted.14,15 Overall, combining the N-cadherin antagonist ADH-1 with LPAM-ILI increased initial response rates but did not significantly alter time to progression at 15 months’ follow-up.12

The objective of this study was to explore the mechanism by which ADH-1 affects the tumor microenvironment, leading to alterations in tumor growth and regional drug delivery. A better understanding of these effects would, in turn, help develop strategies to improve the magnitude and durability of antitumor responses initially observed in phase I and II clinical trials13,15 investigating the safety and efficacy of systemic ADH-1 given before regional cytotoxic LPAM-based therapy. We report data suggesting that systemic ADH-1 has a dual function both (1) to affect vascular permeability in the tumor microenvironment and (2) to modulate tumor growth through activation of the AKT pathway.
MATERIALS AND METHODS

Tumor Cell Lines

The melanoma cell line DM443 was obtained courtesy of Dr. Hilliard Seigler (Duke University, Durham, NC). The A375 cell line was purchased from American Type Culture Collection (ATCC). Cells were maintained as a monolayer in Iscove's modified Dulbecco's medium with 10% fetal bovine serum, 2 mM glutamine, 1000 IU/mL of penicillin, and 100 mg/mL of streptomycin and grown at 37°C, 98% relative humidity, and 5% CO2.

Drugs for Xenograft Therapeutic Studies

LPAM was purchased from Sigma-Aldrich (St Louis, MO). A 0.2-mg/mL stock solution of LPAM was prepared in 0.9% sodium chloride using sonification for dissolution. A 4-mg/mL stock solution of temozolomide (TMZ) was prepared in phosphate-buffered saline with 10% DMSO. Stock solutions of drugs were prepared immediately before surgery. The ILI infusion was prepared by further dilution of TMZ stock solution with a 10% DMSO solution to achieve a final infusion concentration of 2000 mg/kg in a volume of 22.5 mL. Likewise, the LPAM stock solution was further diluted with a 0.9% sodium chloride solution to achieve a final infusion concentration of 90 mg/kg in a volume of 22.5 mL. ADH-1, a pentapeptide that disrupts N-cadherin interactions, was provided by Adherex Technologies, Inc (Research Triangle Park, NC). ADH-1 was prepared in phosphate-buffered saline, and 10 mL/kg of body weight was given via intraperitoneal injection (final dose, 100 mg/kg).

Xenograft Studies

Xenograft studies were conducted as previously reported (see Supplemental Digital Content Methods, available at http://links.lww.com/SLA/A541).13,16,17

Growth Kinetics

Tumor growth was quantified as fold change in tumor volume from the day of ILI. Growth rate (R) was determined from the slope of tumor growth curves during the exponential growth phase. For DM443 xenografts, this was calculated as the slope between days 0 and 30; for A375, this was calculated to be between days 12 and 36. Assuming constant R, doubling time (N) was calculated using the following formula:

\[ N = (t_2 - t_1) \times \frac{\log 2}{\log \left( \frac{q_2}{q_1} \right)} \]

where \( t_1 \) and \( t_2 \) represent the days and their representative fold change in tumor values \( q_1 \) and \( q_2 \).

In Vitro Endothelial Cell Permeability Assay

Human umbilical vein endothelial cells (HUVECs, ATCC) were grown in F-12K base medium supplemented with 0.1 mg/mL of heparin, 0.03 mg/mL of endothelial growth supplement, and 10% fetal bovine serum. HUVECs were grown to confluence on fibronectin-coated Transwell inserts (20 ng/mL of fibronectin per 6.5-mm Transwell insert with a 0.4-μm pore) in normal growth media. Diffusion of 40-kDa fluorescein isothiocyanate (FITC)-conjugated dextran (Sigma) through the HUVEC monolayer was measured after treatment with ADH-1 (1 mg/mL) or VEGF (10 ng/mL) for 1 hour in the presence or absence of serum and growth factors. Diffusion of 40-kDa FITC-dextran (1 mg/mL) across the HUVEC monolayers coating the Transwell insert into the lower compartment was measured using a microplate reader 30 minutes after the addition of FITC-dextran to the Transwell insert.

Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction of N-cadherin Gene Transcripts

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol and including a genomic DNA elimination step. Using 800 ng of RNA, first-strand cDNA synthesis was achieved using the AffinityScript QPCR cDNA synthesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Using a Mx3005P PCR instrument (Stratagene, La Jolla, CA), 100 ng of cDNA was amplified using Brilliant II SYBR-Green qPCR Master Mix (Stratagene) in a 25-μL reaction volume. The mixture was heated at 95°C for 10 minutes, and 40 cycles of 95°C (30 seconds), 55°C (60 seconds), and 72°C (30 seconds) were run, followed by 1 cycle of 95°C (60 seconds), 55°C (30 seconds), and 72°C (30 seconds). The following primers were used: 5′-CCTGGAACCGAGTGTACAGA-3′ and 5′-CTAACCCTGGTGGCCTTGGTCTT-3′ (N-cadherin), and 5′-AAGAA GTGTTGGAAGCGAG-3′ and 5′-GGTGGCGTGTGAAGTCAGA-3′ (GAPDH). The ΔCt values were obtained by normalizing N-cadherin expression to GAPDH expression, which was coamplified in a parallel reaction, to adjust for differences in both the amount of total RNA and different polymerase reaction efficiencies. Controls lacking RNA template were used to monitor for fluorescent contaminants and nonspecific amplification. HUVEC N-cadherin expression is reported as a function of N-cadherin expression for DM366, a high N-cadherin-expressing melanoma cell line.13

Immunofluorescence and Immunohistochemistry

Rats were treated with ADH-1 (100 mg/kg intraperitoneally) or saline 1 hour before ILI and then euthanized 24 hours after LPAM, TMZ, or saline ILI. Tumors were then fixed with formalin, paraffin-embedded, and stained using immunohistochemistry as previously described.13,18,19

Evans Blue Dye Assay

Melanoma cells were injected as described earlier and grown until tumor diameter of 2 cm at which time ILI was performed 1 hour after intraperitoneal injection of 100 mg/kg of ADH-1. A 15-minute infusion of Evans blue dye solution (50 mg/kg dissolved in normal saline and infused at 1.5 mL/min) was followed by a 2-minute saline washout (3.0 mL/min). Animals were then euthanized, and tumors were excised and incubated in formamide solution (72 hours at 37°C). Absorbance of formamide-containing Evans blue dye was measured at 595 nm and normalized to tumor volume.

Tumor Interstitial Fluid Pressure

Similar to permeability studies, melanoma cells were injected as described earlier and grown until tumor diameter was approximately 2 cm3, at which time ILI was performed 1 hour after intraperitoneal injection of 100 mg/kg of ADH-1. After induction of general anesthesia using isoflurane, tumor interstitial fluid pressure (IFP) measurements were carried out with a needle probe pressure monitor (Intra-Compartmental Pressure Monitor System, Stryker), fitted with an 18-gauge side-port needle (Stryker) and connected to a syringe filled with 0.9% saline. The needle probe was inserted into the center of the tumor on the hind limb of the rat. IFP was recorded in millimeters of mercury (mm Hg) when the measurement stabilized. Three to five rats were used for each treatment group.

Reverse-phase Protein Array Analysis

Proteins were isolated from DM443 and A375 xenografts treated with systemic saline or ADH-1 as previously described19
and homogenized. Reverse-phase protein array (RPPA) analysis was performed by the MD Anderson Cancer Center Functional Proteomics Core Facility (see the Web site [http://www.mdanderson.org/education-and-research/resources-for-professionals/scientific-resources/core-facilities-and-servicesfunctional-proteomics-rppa-core/index.html, for detailed methodology and list of antibodies used including source and catalog number]. Differences in relative protein loading were determined and adjusted by using the median protein expression for each sample across all measured proteins utilizing data that had been normalized to the median value of each protein. The values of log2 were converted to linear values, and differences in protein expression between saline- and ADH-1–treated tumors were determined.

**Statistical Analysis**

Overall tumor response to chemotherapy was assessed by comparing fold change in tumor volume at 40 days from ILI. Analysis of variance was used to perform statistical comparisons. A 2-tailed Student t test was used to compare differences in tumor growth rates between treatment arms, permeability, and IFP among xenografts. *P values of less than 0.05 were considered significant.

**TABLE 1. Tumor Volume and Growth Kinetics Summary**

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Systemic</th>
<th>ILI</th>
<th>Fold Δ Tumor Volume*</th>
<th>Growth Rate, per Day†</th>
<th>Doubling Time, d</th>
<th>*P</th>
<th>†P</th>
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<td>DM443</td>
<td>Saline</td>
<td>Saline</td>
<td>11.0 ± 3.0</td>
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<td>11.4 ± 3.1</td>
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<td>ADH-1</td>
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<td>9.2 ± 1.9</td>
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<td>12.5 ± 2.5</td>
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<td>Saline</td>
<td>LPAM</td>
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<td>0.12 ± 0.01</td>
<td>14.8 ± 3.6</td>
<td>0.10</td>
<td>Ref</td>
</tr>
<tr>
<td></td>
<td>ADH-1</td>
<td>LPAM</td>
<td>3.7 ± 0.5</td>
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<td>Saline</td>
<td>TMZ</td>
<td>7.6 ± 0.7</td>
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<td>TMZ</td>
<td>4.4 ± 0.5</td>
<td>0.08 ± 0.01</td>
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<td>0.012</td>
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<td>A375</td>
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<td>Saline</td>
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<td>0.27 ± 0.02</td>
<td>12.6 ± 2.4</td>
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<td>19.9 ± 8.1</td>
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*Calculated 40 days after ILI.
†Two-tailed t test comparing mean growth rate of the treatment group with mean growth rate of control animals treated with systemic saline followed by regional saline infusion.
‡Two-tailed t test comparing mean growth rate after combination treatment of ADH-1 and ILI with LPAM or TMZ to animals receiving ILI with LPAM or TMZ alone.

**FIGURE 1.** DM443 and A375 xenografts treated with systemic ADH-1, followed by ILI with LPAM or TMZ. A, Growth curves of DM443 xenografts treated with systemic ADH-1 or saline, followed by regional infusion of LPAM or saline. B, Growth curves of A375 xenografts treated with systemic ADH-1 or saline, followed by regional infusion of LPAM or saline. C, Growth curves of DM443 xenografts treated with systemic ADH-1 or saline, followed by regional infusion of TMZ or saline. D, Growth curves of A375 xenografts treated with systemic ADH-1 or saline, followed by regional infusion of TMZ or saline. *Analysis of variance of mean fold change in tumor volume at 40 days.
did not significantly decrease tumor growth (0.10 \pm 0.04 per day, \( P = 0.26 \)). In contrast, A375 xenografts treated with systemic ADH-1 followed by arterial saline infusion demonstrated a dramatic and statistically significant and nearly 2-fold increase in growth rate as compared with those receiving both systemic and regional saline (0.53 \pm 0.07 per day vs 0.27 \pm 0.02 per day; \( P = 0.018 \)).

ADH-1 Augmentation of Melanoma Tumor Growth Is Overcome Through Its Ability to Make Regionally Infused LPAM More Effective

As compared with control tumors exposed to both systemic saline and regional saline, LPAM ILI after systemic administration of saline did not significantly alter the mean tumor growth of DM443 xenografts (0.12 \pm 0.01 per day vs 0.20 \pm 0.04 per day; \( P = 0.10 \)) but was associated with a significant decrease in mean tumor growth rate for A375 (0.11 \pm 0.05 per day vs 0.27 \pm 0.02 per day; \( P < 0.001 \)). Combination treatment with systemic ADH-1 followed by regional LPAM infusion led to a further improvement in tumor response for both xenografts. Using the DM443 xenografts, mean tumor growth rates decreased by 75% (0.03 \pm 0.01 per day vs 0.12 \pm 0.01 per day; \( P < 0.001 \)) after regional LPAM treatment (Fig. 1A). Although ADH-1 dramatically augmented tumor growth of A375 xenografts in the absence of LPAM, treatment using regional LPAM concurrently with ADH-1 counteracted this effect. Combination therapy using systemic ADH-1 and regional LPAM decreased the growth rate of A375 tumors by 99% as compared with animals treated with systemic saline followed by regional saline (7.1 e^{-4} \pm 0.02 per day vs 0.27 \pm 0.02 per day; \( P = 0.005 \)). Absolute growth rates also decreased by 99% in the combination ADH-1 and LPAM ILI arm as compared with LPAM ILI alone (7.1 e^{-4} \pm 0.02 per day vs 0.11 \pm 0.05 per day), but this difference did not reach statistical significance (\( P = 0.27 \)). (Fig. 1B). Of note, 80% of animals (4/5) bearing A375 xenografts demonstrated a complete tumor response after combination therapy with ADH-1 and LPAM ILI as compared with only 33% of animals (2/6) treated with LPAM ILI alone.

ADH-1–Mediated Augmentation of Melanoma Tumor Growth Is Not Altered by Regionally Infused TMZ

ILI with TMZ is currently being tested for safety in a phase I/II clinical trial for patients who have progressed after standard ILI with LPAM. Thus, we also tested whether ADH-1 would augment regionally infused TMZ for both DM443 and A375 xenografts in the same series of experiments. For DM443 xenografts, regional infusion of TMZ after systemic administration of saline injection significantly decreased tumor growth rate by 40% (0.12 \pm 0.02 per day vs 0.20 \pm 0.04 per day; \( P = 0.016 \)) as compared with xenografts treated concurrently with systemic and regional saline controls (Fig. 1C). The combination of systemic ADH-1 and regional TMZ led to a 33% reduction in DM443 tumor growth over ILI with TMZ alone (0.08 \pm 0.01 per day vs 0.12 \pm 0.02 per day; \( P = 0.012 \)) and a 40% decrease in tumor growth compared with the systemic and regionally treated saline control animals (0.08 \pm 0.01 per day vs 0.12 \pm 0.01 per day; \( P = 0.009 \)). For A375 tumors, ILI with TMZ demonstrated similar efficacy as compared with ILI with LPAM (56.4% vs 48.6% reduction in tumor growth as compared with the control arm) (Table 1). However, in the presence of ADH-1, TMZ ILI had little efficacy and could not overcome the ADH-1–mediated growth augmentation seen in the A375 xenografts. The combination therapy of systemic ADH-1 plus TMZ ILI had a tumor growth-enhancing effect closer to that seen with systemic ADH-1 alone as compared with the xenografts treated with saline as the systemic and regional control arm (Fig. 1D).

RESULTS

Systemic ADH-1 in the Absence of Regional Chemotherapy Can Both Inhibit and Augment Melanoma Tumor Growth

To explore the mechanisms underlying the variable tumor responses seen in clinical trials testing the combination of N-cadherin antagonism and regional chemotherapy, we first elaborated on previous xenograft growth studies $^{13}$ to include the low N-cadherin–expressing DM443 and high N-cadherin–expressing A375 melanoma cell lines. In vitro, the DM443 cell line is relatively resistant to LPAM and TMZ whereas the A375 cell line is more sensitive to both chemotherapeutic agents than to DM443. $^{19}$ Mean fold change in tumor volumes, growth rates, and doubling times are summarized in Table 1. For DM443, systemic ADH-1 followed by saline infusion did not significantly decrease tumor growth (0.10 \pm 0.04 per day vs 0.20 \pm 0.04 per day, \( P = 0.26 \)). In contrast, A375 xenografts treated with systemic ADH-1 followed by arterial saline infusion demonstrated a dramatic and statistically significant and nearly 2-fold increase in growth rate as compared with those receiving both systemic and regional saline (0.53 \pm 0.07 per day vs 0.27 \pm 0.02 per day; \( P = 0.018 \)).
Tumor Growth Augmentation Mediated by N-cadherin Antagonism Is Associated With Increased AKT Phosphorylation

Multiple studies have associated increased activity of phosphatidylinositol-3 kinase (PI3K)-AKT pathway with melanoma progression and metastasis.\textsuperscript{22,24–26} PI3K activation via receptor tyrosine kinases such as the EGFR (epidermal growth factor receptor) activates AKT, a serine-threonine kinase that regulates the activity of a number of cellular effectors, including the mammalian target of rapamycin (mTOR) protein, leading to increased tumor cell proliferation, invasion, and survival.\textsuperscript{27–29} Previously, we have shown differential phosphorylation of AKT after systemic ADH-1 and regional LPAM ILI.\textsuperscript{13} Moreover, N-cadherin expression has been associated with increased AKT activity in other tumor models.\textsuperscript{30} On the basis of these previous reports, we hypothesized that blocking N-cadherin interactions would result in differential activation of the AKT pathway in A375 and DM443 xenografts. Exponentially growing DM443 and A375 cells were treated with 0 to 1 mg/mL of ADH-1 and analyzed for AKT phosphorylation and PTEN expression. Treatment with ADH-1 was associated with increased AKT phosphorylation in PTEN-expressing A375 cells but did not affect AKT activation in the PTEN-null DM443 cells (Fig. 2A). Similarly, tumor samples from DM443 and A375 xenografts isolated after treatment with systemic ADH-1 or saline were examined using Western blot analyses\textsuperscript{20} to investigate whether ADH-1 treatment alters AKT activation. In A375 but not DM443 xenografts, ADH-1 treatment increased phosphorylation of AKT at serine 473. N-cadherin expression seemed to be slightly diminished in both xenografts after treatment with ADH-1 (Fig. 2B).

RPPA Analysis of the PI3K-AKT-mTOR Pathway

To expand our analysis of the effects of ADH-1 treatment on signaling pathways, we performed RPPA analysis on the A375 and DM443 xenografts. For each xenograft, the effects of ADH-1 were determined by measuring the differences in protein expression levels after ADH-1 treatment for 1 hour as compared with those seen with saline treatment for the same duration. We first analyzed the results for additional members of the PI3K-AKT pathway (Fig. 3A). ADH-1 treatment on the DM443 xenografts resulted in decreased expression of multiple phosphoproteins in the PI3K-AKT pathway, including P-EGFR, P-STAT3, P-STAT5, P-MEK1/2, P-p65, NF-kB, P-FOXO, P-BAD, P-P70S6K, P-4EBP1, S65, and P-GSK3. On the other hand, ADH-1 treatment on the A375 xenografts resulted in increased expression of P-AKT, P-S6, P-P70S6K, P-mTOR, P-4EBP1, S65, and P-GSK3. These results suggest that ADH-1 treatment differentially affects the PI3K-AKT pathway in A375 and DM443 xenografts, with A375 cells showing increased AKT activation and DM443 cells showing decreased AKT activation.

\textbf{FIGURE 3.} Comparative effects of ADH-1 treatment on signaling pathways in the A375 and DM443 xenografts. RPPA analysis was performed on xenografts of A375 (black bars) and DM443 (gray bars) harvested 1 hour after treatment with saline or ADH-1 as outlined in Figure 1. The effects of ADH-1 treatment were determined by subtracting the exponential value of the relative expression for each of the indicated proteins after saline treatment from the observed expression after ADH-1 treatment. Proteins with a relative change of “>0” increased with ADH-1 treatment; “<0” indicates inhibition with ADH-1 treatment. A, Expression of total and phosphoproteins in the PI3K-AKT pathway. B, Expression of total and phosphoproteins in other prosurvival kinase signaling pathways.
ability. On the basis of these data, we measured permeability normal embryonic angiogenesis and for controlling vascular permeability expressed in endothelial cells and has been shown to be critical for morph microenvironment. Along with VE-cadherin, N-cadherin is co-expressed in HUVECs after treatment with saline, ADH-1, or VEGF (positive control). Pretreatment of HUVECs with ADH-1 resulted in an approximate 2-fold increase in dextran permeability compared with saline controls (Fig. 4).

To determine whether the increased expression of activation-specific markers in the PI3K-AKT pathway reflected a general increase in proliferative signaling in the A375, we also examined activation-specific markers in several other pathways (Fig. 3B). ADH-1 treatment inhibited the expression of activation-specific markers in multiple prosurvival pathways in the A375 xenograft to a similar or greater extent than what was observed in the DM443 xenograft, including phosphorylated EGFR, STAT3, STAT5, MEK1/2, and p65. In turn, available total protein levels for the corresponding proteins did not show significant inhibition or notable differences when comparing the 2 xenografts.

Blocking N-cadherin Interactions Increases Endothelial Cell Permeability

Previously, we have shown that systemic ADH-1 increases LPAM-DNA adduct formation after LPAM ILI, suggesting that ADH-1 was increasing drug delivery through alterations in the tumor microenvironment. Along with VE-cadherin, N-cadherin is co-expressed in endothelial cells and has been shown to be critical for normal embryonic angiogenesis and for controlling vascular permeability. On the basis of these data, we measured permeability of the N-cadherin–expressing HUVECs after treatment with saline, ADH-1, or VEGF (positive control). Pretreatment of HUVECs with ADH-1 resulted in an approximate 2-fold increase in dextran permeability compared with saline controls (Fig. 4).

FIGURE 4. Disrupting N-cadherin interactions increases endothelial cell permeability. N-cadherin–expressing HUVECs were grown to confluence in Transwell plates and tested for changes in permeability in response to ADH-1 or VEGF. In the presence and absence of serum and growth factors, pretreatment of endothelial cells (HUVECs) with ADH-1 nearly doubled their permeability to a 40-kDa FITC-conjugated dextran compared with saline controls. This increase in permeability in response to ADH-1 even exceeded the effects of VEGF, used as a positive control for changes in endothelial cell permeability. N-cadherin expression, normalized to GAPDH, in HUVECs is shown with respect to N-cadherin expression in the melanoma cell line DM366, a high N-cadherin–expressing melanoma cell line. GF indicates growth factor.

Targeting N-cadherin In Vivo Transiently Increases Vascular Permeability to Protein-bound Molecules Without Affecting IFP

We investigated whether increased delivery of LPAM seen after systemic ADH-1 treatment resulted from alterations in vascular permeability to protein-bound molecules. Using immunofluorescent techniques, we first confirmed N-cadherin to be expressed in the endothelium of DM443 and A375 xenografts (see Supplemental Digital Content Figure 2a,b, available at http://links.lww.com/SLA/A542). Next, we performed ILI on rats bearing DM443 and A375 xenografts using Evans blue dye as the infused dye 1 hour after treatment with systemic saline or ADH-1. Spectrophotometric analysis of extracted Evans blue dye demonstrated a 28.4% and 32.8% increase in extravasation into DM443 and A375 tumors, respectively, after treatment with ADH-1 (Figs. 5A, B).

Alterations in tumor vascular permeability have been associated with changes in tumor IFP, which have been demonstrated to affect optimal drug delivery to tumor cells. Thus, we measured tumor IFP 1 hour after administration of ADH-1 for both DM443 and A375 xenografts. One hour after administration of ADH-1, no significant differences were found in tumor IFP (Figs. 5C).

ADH-1 Increases Drug Delivery of Regionally Infused LPAM But Not TMZ

We have previously reported that systemic ADH-1 treatment before LPAM ILI can augment delivery of regionally infused LPAM to tumor tissues. Therefore, we tested whether ADH-1 could also enhance delivery of regionally infused TMZ, which is currently under investigation as an alternative regional chemotherapy agent for treatment of advanced extremity melanoma. Rats bearing A375 or DM443 xenografts were systemically treated with ADH-1 or saline control and then infused with regional LPAM or TMZ. Consistent with our previous reports, rats treated with ADH-1 before ILI had demonstrated an approximately 12- and 16-fold increase in LPAM-DNA adducts (surrogate for LPAM delivery) in DM443 and A375 xenografts, respectively (Figs. 6A, B). However, immunohistochemical staining of p-H2A.X (surrogate for TMZ delivery) failed to demonstrate any significant changes in the levels of DNA damage in xenografts treated with ADH-1 before TMZ ILI (Figs. 6C).

DISCUSSION

Here, we report that targeting N-cadherin interactions can independently produce dichotomous effects on melanoma tumor growth in vivo (Table 2). When combined with a high-dose regional LPAM infusion, the contrasting effects of N-cadherin antagonism on tumor growth were abrogated likely by improved drug delivery to the tumor cells resulting from an increase in vascular permeability. However, with the A375 xenograft, ADH-1 not only augmented tumor growth but also failed to increase the cytotoxic effects of TMZ. The net effect led to a TMZ ILI being more effective than a TMZ ILI in combination with ADH-1. Taken together, these studies and previous work in our laboratory suggest 2 separate and independent effects of N-cadherin antagonism in modulating regional chemotherapy: (1) ADH-1 may affect tumor growth and sensitivity to some chemotherapy agents by altering AKT activation and (2) ADH-1 can improve the drug delivery of certain cytotoxic agents such as LPAM by increasing vascular permeability.

We have previously shown that blocking N-cadherin interactions with the pentapeptide ADH-1 before ILI with LPAM results in increased staining of LPAM-DNA adducts in tumors analyzed by immunohistochemistry. Although exhibiting a dichotomous effect on tumor growth, the N-cadherin–targeted agent ADH-1 increased vascular permeability without altering interstitial tumor pressure in

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FIGURE 5. ADH-1 increases Evans blue dye extravasation but not IFP in melanoma xenografts. A, Immunofluorescent analysis was used to determine the extent of N-cadherin expression on A375 and DM443 xenografts. For both xenografts, N-cadherin (green staining) was noted to be readily present on tumor cells and tumor endothelium (white arrow). B, One hour after intraperitoneal injection of 100 mg/kg of ADH-1, ILI was performed on tumor-bearing rats, using Evans blue dye as the instillate (3–5 rats per treatment group). After infusion, animals were sacrificed and tumors were excised and incubated in formamide for 72 hours at 37°C to extract the Evans blue dye into solution. The absorbance of Evans blue dye was then measured in formamide at 595 nm and normalized to tumor volume. Error bars represent the standard error of the mean of 4 to 6 rats. Dye extravasation increased 28.4% from saline control in DM443 (P = 0.0001, t test) and 32.4% in A375 xenografts (P = 0.0001, t test) after treatment with ADH-1. C, One hour after the intraperitoneal injection of 100 mg/kg of ADH-1 or saline control, tumor IFP was measured in DM443 and A375 rat xenografts (3–5 rats per treatment group). Treatment with ADH-1 did not significantly alter tumor IFP for either xenograft model.
ADH-1 May Augment or Inhibit Melanoma Growth

FIGURE 6. ADH-1 improves delivery of LPAM but not TMZ. Using our animal model of ILI, rats were pretreated with ADH-1 1 hour before infusion of LPAM or TMZ. LPAM and TMZ drug delivery after ILI was assessed using immunohistochemical staining of LPAM-DNA adducts (MP5 antibody) and DNA damage (p-H2A.x antibody) as surrogates for LPAM and TMZ delivery, respectively. A, Qualitatively, ADH-1 increased LPAM-DNA staining for both xenografts. B, LPAM-DNA adduct staining was then quantified digitally as the ratio of brown to total pixels. As compared with saline controls, pretreatment with ADH-1 resulted in an approximately 12- and 16-fold increase in LPAM-DNA adduct formation for both xenografts. C, In contrast, pretreatment of ADH-1 before TMZ ILI revealed no significant changes in the amount of DNA damage. D, Quantification of immunohistochemical staining for p-H2A.x suggests a statistically nonsignificant decrease in TMZ delivery after ADH-1 treatment. IA indicates inter-arterial; IP, inter-peritoneal.

both xenografts 1 hour after a single injection. How might the increased vascular permeability resulting from disrupting N-cadherin-binding interactions improve the efficacy of LPAM but not TMZ? Tumor angiogenesis is notoriously rapid and abnormal, resulting in tumor vasculature that is saccular, tortuous, and leaky resulting in abnormal pressure gradients and inefficient drug delivery. The enhanced permeability and retention effect, which results from this abnormal vasculature, states that drug-transporting macromolecules preferentially extravasate and accumulate into tumor tissues as opposed to normal tissues where intact tight junctions of endothelial cells prevent leakage of such drug carriers. Furthermore, the increase in tumor vascular permeability occurred in the absence of tumor IFP changes. Thus, if the increased drug delivery seen in our study is indeed manifested by the enhanced permeability and retention effect, it would be not be hindered from an increase in IFP that is often seen with increased tumor vascular permeability. Sixty percent to 90% of all LPAM is bound to plasma proteins, whereas TMZ is lipophilic and not known to undergo any specialized membrane transport processes. Therefore, it is conceivable that ADH-1-mediated alterations in vascular permeability may allow greater accumulation of drugs into the interstitium, providing active transporters more time to uptake LPAM while not affecting the passive diffusion through which TMZ or its active metabolite MTIC enter tumor cells.

TABLE 2. Summary of ADH-1 Drug Effects in a Regional Therapy Model of In-transit Melanoma

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DM443 Effect</th>
<th>A375 Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenograft growth</td>
<td>No change</td>
<td>Increased</td>
</tr>
<tr>
<td>LPAM ILI†</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>TMZ ILI†</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>AKT activation</td>
<td>No change</td>
<td>Increased</td>
</tr>
<tr>
<td>Vascular permeability</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>LPAM delivery</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>TMZ delivery</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

*Growth rate of animals treated with combination therapy (ADH-1 + LPAM ILI or TMZ ILI) as compared with the mean growth rate of control animals treated with systemic saline followed by saline ILI.

†Xenograft growth in rats treated with systemic ADH-1 followed by saline ILI as compared with control rats treated with systemic saline followed by saline ILI.
The ability of ADH-1 to both inhibit and augment melanoma tumor growth in vivo seems to be independent of its effect on the tumor vasculature, suggesting N-cadherin to have heterogeneous roles in regulating melanoma tumor signaling potentially dependent on the magnitude of other molecular and signaling alterations within the tumor. To provide insight into the differential effects of ADH-1, we performed proteomic analysis of ADH-1–treated xenografts by Western blotting and RPPA analysis. This analysis demonstrated that despite the increased growth observed, treatment of the A375 xenografts with ADH-1 was associated with decreased expression of activation-specific markers of multiple signaling pathways, including the JAK-STAT, RAS-RAF-MEK-ERK, and NFκB pathways (Fig. 6B). In contrast, although treatment with ADH-1 seemed to also inhibit the PI3K-AKT pathway in the sensitive DM443 xenograft, we observed increased expression of multiple activation- (phospho-) specific and total protein levels of critical effectors of this pathway in the resistant A375 xenograft. ADH-1 treatment also resulted in decreased expression of PTEN in the A375 xenograft, which has been shown to correlate with increased activation/phosphorylation of multiple kinase(s) in this pathway, particularly AKT, in melanoma previously.22 It should be noted, however, that the alterations seen in these signaling pathways after ADH-1 treatment may be purely associative and may not necessarily be a direct result ADH-1 binding to N-cadherin.

The “double-edged sword” effect reported here after N-cadherin antagonism has been seen in other settings. For instance, angiogenesis inhibitors in preclinical studies have been shown to eventually increase local invasion and metastasis by selecting for a tumor clonal population with hypoxia tolerance and promoting rescue angiogenesis.45,46 More recently, PLX4032 (vemurafenib), a novel inhibitor of the activating V600E BRAF mutation seen in approximately 60% of melanomas, has been shown to be marked effectively in a phase III trial of metastatic melanoma. For patients harboring the V600E BRAF mutation, 48% treated with PLX4032 responded as compared with only 5% of patients treated with the standard chemotherapeutic agent dacarbazine.47 Despite high responses to PLX4032, 21% of patients eventually developed cutaneous squamous cell carcinomas after initiating treatment. Further research suggests that V600E BRAF mutant inhibitors such as PLX4032 paradoxically activate downstream signaling (ie, MEK-ERK) in both transformed and nontransformed cells lacking the BRAF mutation and may be responsible for the development of cutaneous squamous cell carcinoma.48–50

Given that ADH-1 has already been studied in combination with LPAM II in phase I and II clinical trials, this study reports findings of significant clinical relevance. With these trials, no evidence of rapid growth or disease progression was observed but patients received only 2 doses of ADH-1 as part of these studies. Here, we report that the response of ADH-1 among different melanoma xenografts is variable, highlighting the need for further investigation to delineate the biological underpinnings for responders and nonresponders. Although ADH-1 may significantly improve delivery of small cytotoxic agents, its disruption of N-cadherin binding may have variable effects on tumor growth based upon the alterations in association with AKT activation. As future trials may be planned targeting N-cadherin, it will be important to understand factors that predict how tumor cells will respond to this targeted intervention. In addition, clinicians will have to be vigilant for potential detrimental tumor-augmenting effects that may occur and are not always identified in preclinical studies that focus on small numbers of tumors.

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REFERENCES


