Targeting N-Cadherin Enhances Antitumor Activity of Cytotoxic Therapies in Melanoma Treatment

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Abstract

Malignant transformation in melanoma is characterized by a phenotype "switch" from E- to N-cadherin, which is associated with increased motility and invasiveness of the tumor and altered signaling, leading to decreased apoptosis. We hypothesized that the novel pentapeptide (ADH-1), which disrupts N-cadherin adhesion, could sensitize melanoma tumors to the cytotoxic effects of chemotherapy. N-cadherin–expressing human melanoma-derived cell lines were used to generate xenografts in animal models to study isolated limb infusion with melphalan and systemic chemotherapy with temozolomide. We report here that melphalan in combination with ADH-1 significantly reduced tumor growth up to 30-fold over melphalan alone. ADH-1 enhancement of response to melphalan was associated with increased formation of DNA adducts, increased apoptosis, and intracellular signaling changes associated with focal adhesions and fibroblast growth factor receptors. Targeted therapy using an N-cadherin antagonist can dramatically augment the antitumor effects of chemotherapy and is a novel approach to optimizing treatment for melanoma. [Cancer Res 2008;68(10):3777–84]

Introduction

The incidence of malignant melanoma is increasing at a rate faster than any other cancer with an expected 60,000 new cases diagnosed this year in the United States (1). Melanoma that has spread to distant sites is rarely curable—treatment options are limited and response rates are poor and short-lived (2, 3). There have been few changes to treatment strategies for these patients in nearly 40 years and expanding therapeutic options will be essential for improving outcomes.

Melanoma is characterized by a switch in expression of the cadherin family of cell adhesion molecules from a largely E-cadherin–expressing phenotype to a predominantly N-cadherin–expressing one (4, 5) as it progresses from a preinvasive to a metastatic form. In normal melanocytes, E-cadherin expression provides for high-affinity anchoring to neighboring keratinocytes and serves as a tumor suppression mechanism (4). With malignant transformation, generally at the vertical growth phase transition, N-cadherin is up-regulated as a dominant phenotype. Transformed melanocytes acquire invasive migratory capabilities, an ability to transit the endothelial vascular barrier and undergo epithelial-to-mesenchymal transition (6–8). Src tyrosine kinase has been shown to be activated during transendothelial migration of melanoma cells, and other intracellular signaling pathways may also be altered by this switch with downstream consequences such as increased proliferation, survival and angiogenesis, and decreased apoptosis (9).

ADH-1 is a novel cyclic pentapeptide that contains the cell adhesion recognition site (His-Ala-Val) important in N-cadherin adhesion. Previous reports showed that peptides containing this sequence disrupt cell adhesion, induce apoptosis, and alter the intracellular distribution of β-catenin and actin in endothelial cells (10). The antitumor effects of N-cadherin disruption with ADH-1 are thought to occur by two mechanisms—altering intracellular signaling pathways coupled to the cadherin adhesion complexes and disrupting heterotypic cell-cell contacts between the tumor cells and surrounding endothelial cells. Although ADH-1 has been well-tolerated in phase I clinical trials, its antitumor efficacy as a single agent has been modest (11, 12). However, given its wide distribution in the majority of metastatic melanoma lesions, the signaling pathways connected to it and the role of N-cadherin adhesion in tumor survival mechanisms, N-cadherin satisfies several of the criteria that would make it an ideal candidate for targeting as a means to sensitize tumor cells to the cytotoxic effects of concurrently administered chemotherapy.

Using a panel of melanoma xenografts, we have studied the effects of ADH-1 on tumor response rates to two chemotherapy reagents. We report here that response to therapy with either regionally administered melphalan or systemically administered temozolomide is enhanced when used in combination with systemic ADH-1. ADH-1 when used in combination with chemotherapy lead to increased apoptosis and DNA adduct formation as well as changes in intracellular signaling associated with focal adhesions, the Abl kinase, and the fibroblast growth factor (FGF) receptor.

Materials and Methods

Tumor cell lines and xenograft studies. Tumor cell lines derived from human malignant melanomas (DM lines) were courtesy of Dr. H. Seigler (Duke University Medical Center, Durham, NC). All other cell lines were purchased from American Type Culture Collection. Cells were cultured in Iscove's modified Dulbecco's medium with 10% fetal bovine serum, 2 mmol/L glutamine, 1,000 IU/mL penicillin, and 100 mg/mL streptomycin and grown at 37°C and 5% CO_2.

Female nude rats (6-wk-old; Charles River Laboratories International, Inc.) were injected s.c. in the right hind limb with 5×10^6 cells. Tumor volume was calculated as: $[(\text{length}) \times (\text{width})^2]/2$. Treatment began when tumor volume was 100 mm^3. The rat protocol was approved by the Duke

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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University and Durham VA Medical Center Institutional Animal Care and Use Committees.

For systemic studies, female nude mice (HRLN nu/nu) were injected s.c. in the right flank with 1×10^7 cells DM366. A375 was maintained in athymic nude mice by serial engraftment; a tumor fragment (1 mm^3) was implanted s.c. into the right flank. On day 1 of the study (tumor volume, ~150 mm^3), mice were assigned to treatment groups. All mouse studies were performed following an approved animal protocol at Piedmont Research Center.

Temozolomide (Temodar; Schering Corp.) and melphalan (Glaxo-Wellcome, Inc.) were purchased. A 10 mg/mL temozolomide stock solution was prepared fresh; 10 mL/kg body weight was given via i.p. injection (final dose, 100 mg/kg). A 22.5 mL isolated limb infusion (ILI) infusate of 0.9% sodium chloride solution containing 90 mg/kg melphalan was prepared fresh. ADH-1, kindly provided by Adherex Technologies, Inc., was prepared in PBS, and 10 mL/kg body weight was given via i.p. injection (final dose, 100 mg/kg).

ILI was performed as described previously (13–15). The femoral artery and vein were cannulated. The arterial catheter was attached to a peristaltic pump while venous drainage flowed by gravity into a reservoir. A tourniquet was placed around the thigh. A 15-minute melphalan infusion (1.5 mL/min) was followed by a 1 min saline wash-out (3.0 mL/min).

Response to drug treatment was followed until tumor volume reached 5,000 mm^3 or 60 d for regional studies, or tumor volume reached 2,000 mm^3 for systemic studies. Response was evaluated by the percent increase in tumor volume, tumor quintupling time, maximal decrease in tumor volume, number of tumor regressions, and response to treatment for 6 (regional melphalan) or 10 (systemic temozolomide) animals per group.

Western blot, immunohistochemistry, and expression analysis. For protein analysis, tumor was lysed in a modified radioimmunoprecipitation assay buffer and analyzed by SDS-PAGE followed by immunoblotting. Antibodies used were as follows: Akt, pAKt (Ser^{473}), phosphorylated Paxillin (pPaxillin; Tyr^{188}), pCrkL (Tyr^{207}), CrkL, and E-Cadherin (Cell Signaling Technology); FGFR-1 (Flg) and cSrc (Santa Cruz Biotechnology, Inc.); N-Cadherin and Paxillin (Zymed Laboratories); Abl and Akt (BD Biosciences); and actin (Sigma-Aldrich). Immunohistochemistry (IHC) was performed as previously described (16, 17). Treated and nontreated melanoma was used as positive and negative control for the MP5/73 antibody (kindly provided by Dr. M.J. Tilby, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; refs. 18–20). Tissue sections stained for Klenow Frag-EL (Oncogene) were stained according to the manufacture's instructions. RNA was isolated using the Qiagen RNeasy mini kit.

Statistical analysis. The tumor response to chemotherapy was assessed by ANOVA. Student's *t* test was performed for tumor regressions. *P* values of <0.05 were considered significant. Correlation coefficients were obtained using a regression analysis.

See Supplementary Data for additional details.

Results

Expression of N- and E-cadherin. Expression of N- and E-cadherin was evaluated across a panel of melanoma cell lines to identify the degree to which these melanomas have undergone a "switch" in cadherin expression (Fig. 1*A*). Gene expression data were confirmed at the level of protein expression across a subset of these melanomas (Fig. 1*B*). In general, there was a strong correlation between relative protein and relative RNA expression when compared across the 13 cell lines evaluated. In some examples (i.e., DM6 and DM646), relative protein expression was lower than expected based on relative RNA expression. This is not unexpected as translational regulation of cadherins is likely to vary

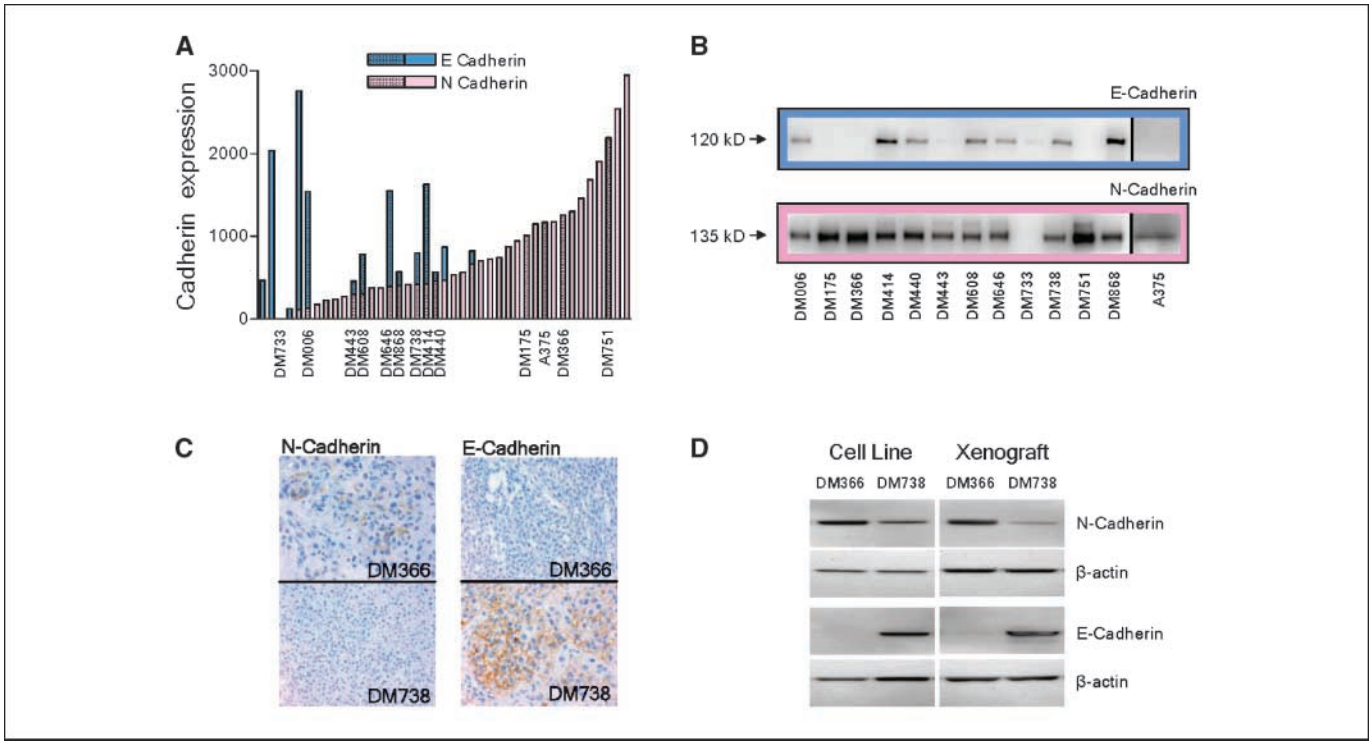

Figure 1. Expression of E- and N-cadherin cell adhesion proteins in melanoma. *A,* gene expression was measured across a panel of 40 human melanoma-derived cell lines using Affymetrix GeneChips. Shown is expression of E-cadherin (*blue bars,* probe sequences derived from accession number NM_004360; probe set ID: 201131_s_at) and N-cadherin (*pink bars,* probe sequences derived from accession number NM_001792; probes set ID: 203441_s_at). Expression values defined as not present were set to a value of 0. Cell lines noted with hatched bars correspond to those in *B. B*, Western Blot showing protein expression of E-cadherin (*top*) and N-cadherin (*bottom*) across 13 of the cell lines from *A. C,* IHC of xenografts derived from human melanomas DM366 (*top*) and DM738 (*bottom*) showing N-cadherin (*left*) and E-cadherin (*right*) expression. *D,* Western Blot showing protein expression of E-cadherin (*bottom*) and N-cadherin (*top*) across DM366 and DM738 cell line (*left*) and xenograft (*right*) samples. β-actin was used as a loading control and is shown below the cadherin blots.

across cells (21). All but one cell line (DM733) expresses N-cadherin, and for three (DM175, DM366, and DM751), only N-cadherin expression was detectable. The observed pattern of cadherin expression is consistent with previous reports demonstrating that loss of E-cadherin with a concomitant gain of N-cadherin is common in melanoma (5, 22). Representative IHC staining of N- and E-cadherin is shown in Fig. 1*C* for two melanoma xenografts—DM738 and DM366. Although N-cadherin expression is clear in xenograft DM366, there was no detectable N-cadherin expression in xenograft DM738 as measured by IHC. Matched Western blots for DM366 and DM738 cell line and xenograft samples (Fig. 1*D*), however, showed clear expression of N-cadherin across all samples. Notably, staining for N-cadherin was lower in xenografts compared with the matched cell line and may represent reduced expression of N-cadherin after s.c. injection and *in vivo* growth. The negative IHC results for DM738 N-cadherin expression may represent reduced sensitivity in IHC measurements compared with direct protein analysis. Results, however, were consistent with protein and RNA analysis in cell lines in that N-cadherin staining was higher in DM366 then in DM738, whereas E-cadherin was higher in DM738 compared with DM366.

Inhibition of N-cadherin binding augments chemotherapy. To study the effects of N-cadherin antagonism, we used an animal model of regional therapy (ILI) for extremity melanoma (15, 23), melphalan, and the treatment protocol illustrated (Fig. 2*A*). ADH-1 alone had a nonsignificant effect on tumor growth rate in DM366, a high N-cadherin–expressing xenograft (Fig. 2*B*). Although DM366 responded well to melphalan, the combination of ADH-1 with melphalan was significantly ($P < 0.005$) more effective (Fig. 2*B* and *C*). We evaluated the extent to which ADH-1 can overcome resistance to melphalan with the xenograft DM738, which does not respond to melphalan alone (Fig. 2*D*). ADH-1 alone had a nonsignificant tumor growth–accelerating effect; however, when ADH-1 was used in combination with melphalan, tumor growth was significantly ($P < 0.005$) slowed. Table 1 summarizes these results. ADH-1 was not cytotoxic or associated with any adverse events (nadir body weight did not exceed −5% from baseline for animals treated with ADH-1 alone). Melphalan in combination with ADH-1 was significantly more effective than melphalan alone when evaluated across multiple variables including percent change in tumor volume, partial and complete response rates, and regression.

To study the effects of N-cadherin antagonism in combination with systemic chemotherapy with temozolomide, we used a mouse model of melanoma. Xenograft DM366 was moderately sensitive to temozolomide alone (Fig. 3*A*). When temozolomide was used in combination with ADH-1 (Fig. 3*B*), there was a slight, not statistically significant improvement in response (Fig. 3*A*; $P = 0.16$). We evaluated the degree to which N-cadherin disruption can overcome resistance to temozolomide with the melanoma xenograft A375, an N-cadherin–expressing (ref. 22; Fig. 1*A*) and temozolomide-resistant xenograft. The treatment protocol (Fig. 3*D*) included three daily ADH-1 doses before initiating chemotherapy with temozolomide. Neither temozolomide nor ADH-1 alone had an effect on tumor growth rate (Fig. 3*C*); however, when temozolomide was used in combination with ADH-1, tumor growth was slowed ($P < 0.05$). These results are summarized in Table 1. As in the regional studies, ADH-1 was not cytotoxic or associated with any adverse events.

Inhibition of N-cadherin binding alters intracellular signaling and increases apoptosis. To identify intracellular signaling changes that could account for these responses to ADH-1, we

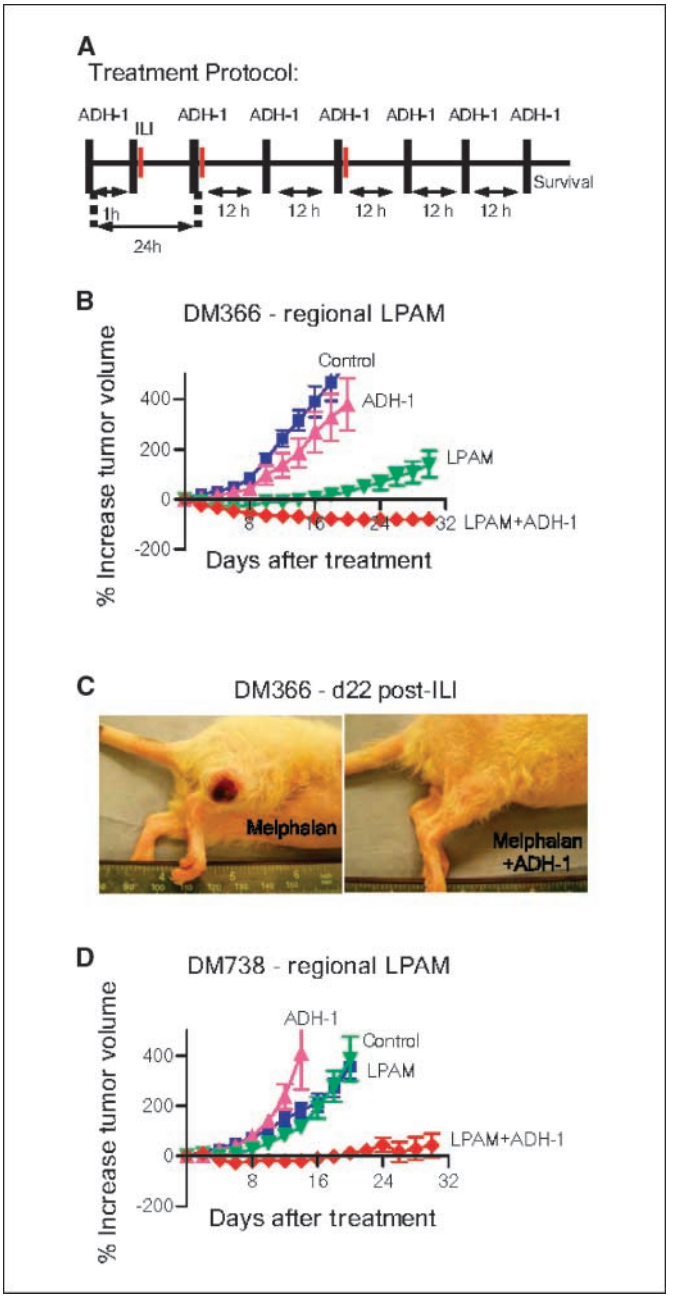

Figure 2. ADH-1 augments response to regional chemotherapy with melphalan. *A,* schematic illustration of regional chemotherapy treatment protocol in combination with systemic ADH-1. An initial dose (100 mg/kg) of ADH-1 was given 1 h before ILI with melphalan (90 mg/kg). A second dose of ADH-1 was given 24 h after the first dose and again every 12 h for an additional 5 doses. Tumor volume was measured every other day for 60 d after ILI. *Red lines,* time points at which tumor was harvested for nonsurvival studies (see Fig. 4). *B,* percent change in tumor volume is plotted as a function of time after ILI with melphalan or saline. In this study, a melphalan-sensitive melanoma, DM366, was studied. ADH-1 or saline control was given i.p. as illustrated in *A*. Control (*blue*); ADH-1 alone (*pink*); melphalan alone (*green*); melphalan plus ADH-1 (*red*). *Points,* mean percent change tumor volume for six animals at each time point; *bars,* SE. *C,* photographs of a representative tumor before and after treatment with ADH-1 in combination with melphalan at day 22 after ILI with melphalan. *D,* percent change in tumor volume plotted as a function of time. In this study, a melphalan-resistant melanoma, DM738, was studied. ADH-1 or saline control was given i.p. as illustrated in *A*. Control (*blue*); ADH-1 alone (*pink*); melphalan alone (*green*); melphalan plus ADH-1 (*red*). *Points,* mean percent change tumor volume for six animals at each time point; *bars,* SE. *LPAM,* melphalan.

performed a series of nonsurvival studies using xenograft DM366 and our animal model of regional ILI with melphalan (Fig. 2*A*). There was a marked increase in apoptosis in melphalan plus ADH-1 compared with melphalan or ADH-1 alone as measured using immunohistochemical staining of fragmented DNA ends (FragEL; Fig. 4*A*). To test whether this increased apoptosis was due to increased formation of DNA adducts, we used the antibody MP5/73, which is specific for melphalan-DNA adducts (18, 20). There was an increase in the number of cells staining positively for adducts at 24 h post-ILI in melphalan plus ADH-1 compared with melphalan alone (Fig. 4*A*). As expected, there were no adducts formed in either saline or ADH-1 alone.

To gain further insight into the mechanism by which ADH-1 in combination with melphalan leads to increases in apoptosis, cell death, and tumor regression, we measured the expression of several signaling proteins 4 h after completion of ILI (Fig. 4*B*). Expression of N-cadherin and β-catenin, which is associated with the intracellular domain of N-cadherin adhesion complexes, did not change in response to treatment (data not shown). Focal adhesion kinase and Src kinase, important in the transduction of signals from sites of cell adhesion (24), showed no change in expression in response to treatment nor did the levels of their phosphorylated forms (data not shown).

N-cadherin functionally interacts with and stabilizes the FGF receptor type 1 (FGFR-1; ref. 25), which can lead to increased survival and invasiveness of the tumor cell (26). Although FGFR-1 expression did not change with either melphalan or ADH-1 alone, it decreased markedly in the melphalan plus ADH-1 treatment group (Fig. 4*B*). The adaptor protein paxillin is important in the linking of signals from integrins and growth factor receptors to actin-cytoskeletal structures and, ultimately, to cell motility and migration (27). Whereas expression of total paxillin did not change in response to treatment, pPaxillin decreased slightly in ADH-1 alone and markedly in melphalan plus ADH-1 (Fig. 4*B*). The adaptor proteins Crk and Crk-like (Crk/CrkL), similar to and often in association with paxillin, play a critical role in the regulation of actin cytoskeleton, cell migration, and survival (28). Phosphorylation of Crk/CrkL is regulated by both Abl kinase (29) and epidermal growth factor receptor–dependent phosphorylation (30). Expression of Abl kinase and the associated Arg kinase did not change across the treatment groups (Fig. 4*B*). Expression of CrkL decreased in the ADH-1 and melphalan plus ADH-1 treatment groups, whereas phosphorylated Crk/CrkL increased slightly in ADH-1 alone and markedly in the melphalan plus ADH-1 treatment groups (Fig. 4*B*). It has been shown that engagement of N-cadherin adhesion complexes can inhibit apoptotic signaling by way of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (31). Although we observed no change in the expression of total Akt, there was a marked increase in phosphorylated Akt (pAkt) in melphalan plus ADH-1 (Fig. 4*B*).

Discussion

We have shown that the pentapeptide ADH-1 can enhance the effectiveness of regional or systemic chemotherapy in melanoma. The mechanism by which this occurs is complex and may involve increased formation of melphalan-DNA adducts and alterations in focal adhesions and FGFR signaling.

In this study, two animal models were used in combination with a panel of melanoma xenografts—a model of regional chemotherapy with ILI of melphalan and a murine model of systemic chemotherapy with temozolomide. The advantage of the regional therapeutic strategy is that modulators, such as the N-cadherin antagonist ADH-1, can be given systemically in combination with high doses of regionally administered chemotherapy. Melphalan, a phenylalanine derivative and alkylating agent, is one of the drugs of choice for regional infusion (32, 33) chemotherapy for in transit, extremity melanoma. Temozolomide

Table 1. Summary of tumor response and toxicity to ADH-1, melphalan, and temozolomide

Treatment:	Regional melphalan					
	DM366			DM738		
	ADH-1	Melphalan	Melphalan + ADH-1	ADH-1	Melphalan	Melphalan + ADH-1
PR Rate (%)	0	25	40	0	0	33
CR Rate (%)	0	0	60	0	0	0
Regression (%)	0	75	100	0	17	100
Nadir body weight (%)	−4.2	−8.2	−10.5	−3.2	−1	−13.2
Treatment:	Systemic temozolomide					
	DM366			A375		
	ADH-1	Temozolomide	Temozolomide + ADH-1	ADH-1	Temozolomide	TEMOZOLOMIDE + ADH-1
PR Rate (%)	0	50	60	0	0	0
CR Rate (%)	0	20	40	0	10	0
Regression (%)	0	70	90	0	0	0
Nadir body weight (%)	−4.3	−9.1	−11.7	0	0	−3.5

Abbreviations: PR, partial response; CR, complete response.

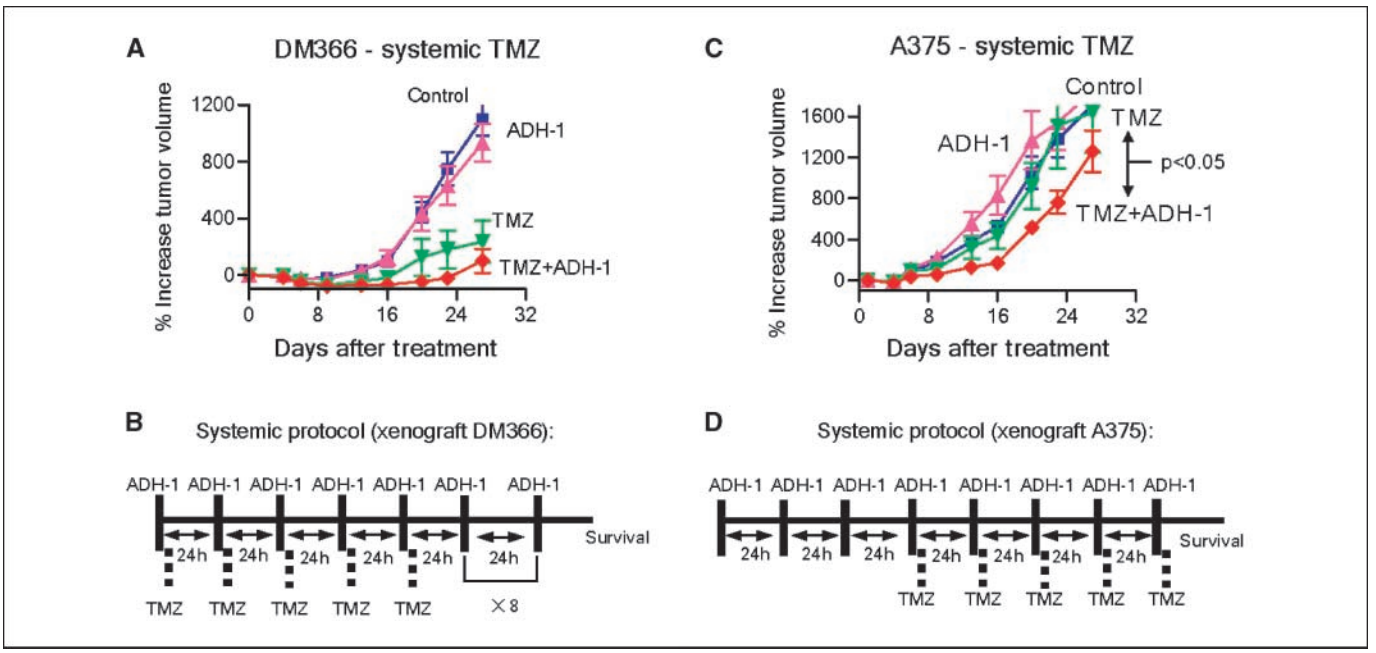

Figure 3. ADH-1 augments response to systemic chemotherapy with temozolomide (*TMZ*). *A,* percent change in tumor volume is plotted as a function of time after systemic treatment with temozolomide or saline. In this study, a temozolomide-sensitive melanoma, DM366, was studied. ADH-1 or saline control was given i.p. as illustrated in *B*. Control (*blue*); ADH-1 alone (*pink*); temozolomide alone (*green*); temozolomide plus ADH-1 (*red*). *Points,* mean percent change tumor volume for 10 animals at each time point; *bars,* SE. *B,* schematic illustration of systemic chemotherapy treatment protocol in combination with systemic ADH-1 used for the study of xenograft DM366. Temozolomide was dosed p.o. once daily (100 mg/kg) for 5 d. ADH-1 (100 mg/kg) was given 1 h before the temozolomide dose. This was repeated once daily for 4 more d (the duration of temozolomide treatment); ADH-1 treatment continued for an additional 8 daily doses. Tumor volume was measured every other day for up to 30 d after the start of the treatment protocol. *C,* percent change in tumor volume is plotted as a function of time after systemic treatment with temozolomide or saline. In this study, a temozolomide-resistant melanoma, A375, was studied. ADH-1 or saline control was given i.p. as illustrated in *D*. Control (*blue*); ADH-1 alone (*pink*); temozolomide alone (*green*); temozolomide plus ADH-1 (*red*). *Points,* mean percent change tumor volume for 10 animals at each time point; *bars,* SE. *D,* schematic illustration of systemic chemotherapy treatment protocol in combination with systemic ADH-1 used for the study of xenograft A375. Three once daily doses of ADH-1 (100 mg/kg) were given before the start of temozolomide treatment. On day 4, ADH-1 was given 1 h before temozolomide (100 mg/kg). This was repeated once daily for 4 more d (the duration of the temozolomide treatment). Tumor volume was measured every other day for up to 30 d after the start of the treatment protocol.

is a methylating and monofunctional alkylating agent with a mechanism of action similar to dacarbazine, currently the most widely prescribed drug for systemic treatment of patients with metastatic melanoma (34). Although not Food and Drug Administration–approved for melanoma temozolomide is increasingly used due to its oral formulation and ability to penetrate the blood brain barrier. Response rates of 13.5% have been reported for temozolomide when used as a single agent (35).

There are several drugs in various stages of clinical trials that selectively target pathways frequently dysregulated in cancer (2). Targeted therapeutics are often not inherently cytotoxic, and although response rates to these drugs alone have been modest, it is likely that when used in combination with cytotoxic chemotherapy, response rates will be enhanced. Our results clearly show the therapeutic potential of combination treatment strategies. ADH-1 was neither cytotoxic nor associated with any adverse events in these studies when used alone or in conjunction with either regionally administered melphalan or systemically administered temozolomide.

ADH-1 alone showed negligible effects on tumor growth in both the DM366 and A375 xenografts when compared with saline-matched controls. We did note a slight, not statistically significant ($P > 0.1$), increase in tumor growth after treatment with ADH-1 alone in the DM738 xenograft. Across all models studied, the therapeutic advantage of ADH-1 came when it was used in combination with chemotherapy. The slight enhancement of DM738 tumor growth with ADH-1, if real, may represent a unique signaling event in response to ADH-1 disruption of N-cadherin adhesion complexes. Future studies will monitor the growth-promoting effects of ADH-1 as a single agent to determine the extent to which it occurs, whether there is a relationship between this response and the molecular properties of the tumor and the nature of the intracellular signaling pathways that confer this response.

The response to ADH-1 in combination with regional chemotherapy with melphalan was more dramatic than the response to ADH-1 in combination with systemic chemotherapy with temozolomide. Comparing responses to chemotherapy in combination with ADH-1 in our two most resistant xenografts, there was a 30-fold reduction in percent change in tumor volume at day 20 when melphalan was used (DM738 xenograft), whereas there was only a 2-fold reduction in this same response measured at day 23 when temozolomide was used (A375 xenograft). The difference in the ability of ADH-1 to enhance responsiveness in chemoresistant tumors may be a function of the properties of each of these xenografts. However, the less chemoresistant xenograft, DM366, which was studied with both chemotherapy agents, likewise showed reduced responsiveness in the setting of systemic chemotherapy with temozolomide (compare Figs. 2*B* and 3*A*). This suggests that the drug (melphalan versus temozolomide) and/or drug delivery (systemic versus regional infusion) may be important factors in the enhanced response to chemotherapy. Doses of chemotherapy given in the regional setting compared with the systemic setting are much higher, and it is possible that better

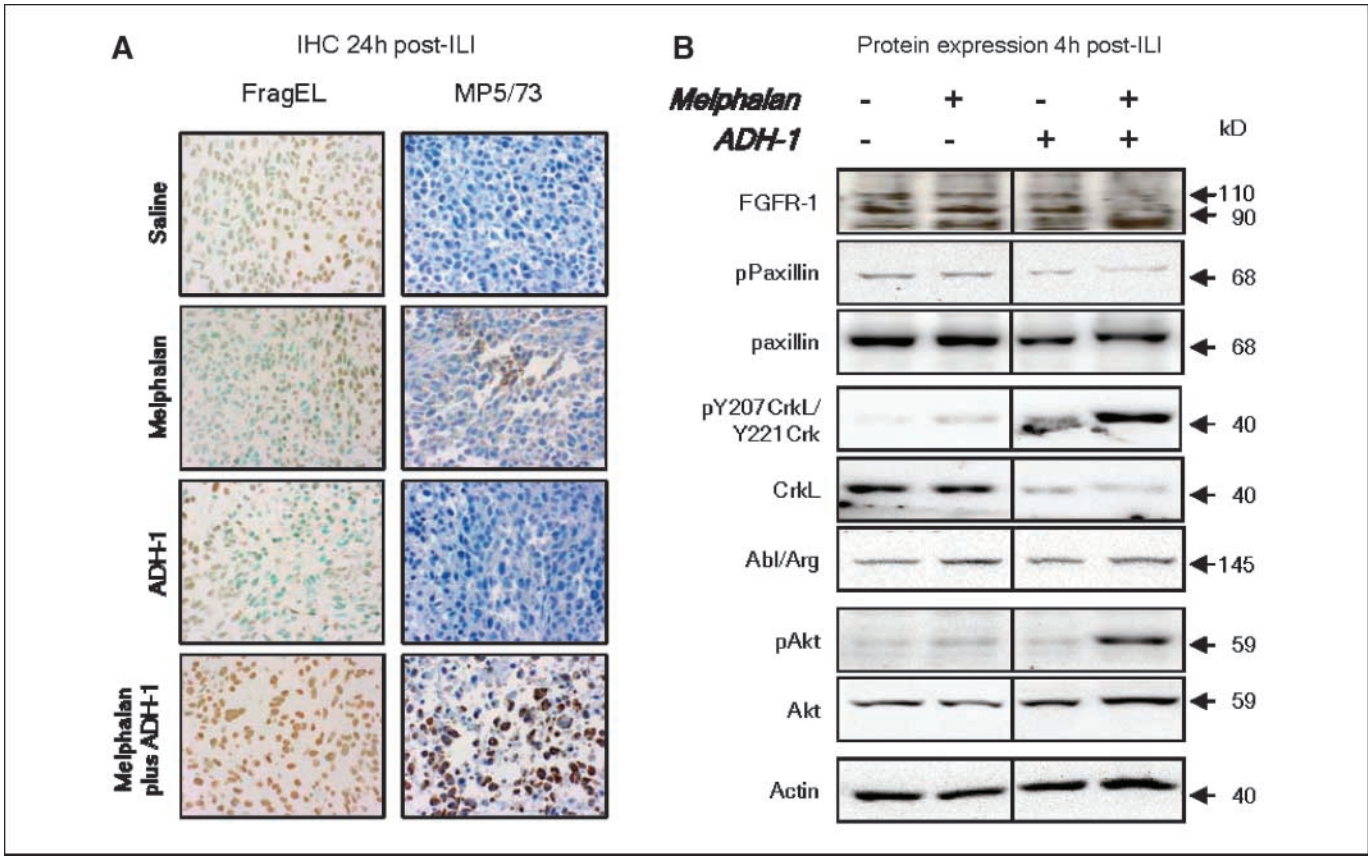

Figure 4. Altered intracellular signaling in response to ADH-1. *A,* IHC of tissue slices taken from saline, melphalan, ADH-1, and melphalan plus ADH-1 treated samples. Apoptosis was measured as the number of FragEL (*left*), whereas melphalan-DNA adducts were measured using the antibody MP5/73 (*right*). The fraction of apoptotic cells (*left*) was higher in the melphalan plus ADH-1 treatment group as evidenced by the prominent staining of FragEL (*brown*) and the nearly total absence of nuclear (*blue,* hematoxylin) staining. DNA adduct formation (*right*) was higher (*brown stain*) in the ADH-1 plus melphalan–treated samples compared with the melphalan alone–treated samples. As expected, no DNA adduct staining was seen in samples that were not exposed to melphalan. *B,* clarified cell lysates (10–20 mg) were analyzed by SDS-PAGE and immunoblotted with the following antibodies: FGFR-1, paxillin, pPaxillin, pY207CrkL/pY221Crk, CrkL, Abl/Arg, pAkt, Akt, and Actin, as a loading control. Bound antibodies were detected using a chemiluminescence visualization kit.

systemic responses might be seen with either higher dosing or repeated cycles of systemic chemotherapy. The mechanism of action of melphalan and temozolomide are different as is cellular uptake where amino acid transport is important for melphalan but not for temozolomide (36)—these factors may effect the effectiveness of ADH-1 in combination with chemotherapy. Further studies will help elucidate the mechanism by which ADH-1 enhances chemotherapy and how this may depend on the chemotherapy agent and mode of delivery.

The cytotoxic actions of melphalan derive from the formation of DNA adducts and subsequent initiation of apoptosis and cell death (37, 38). The increased formation of DNA adducts in the melphalan plus ADH-1–treated xenografts suggests that the increased apoptosis observed in these xenografts was at least in part a consequence of enhanced adduct formation. Potentially contributing further to the enhanced cytotoxic action of melphalan were changes in several signaling proteins. CrkL and Crk are downstream targets of Abl/Arg nonreceptor tyrosine kinases (39, 40), and the increased phosphorylation of the CrkL/Crk proteins we observed suggests an increase in the activity of Abl or Arg kinase. Downstream consequences of the increased phosphorylation in CrkL/Crk proteins are complex but could include inhibition of cell migration and survival, disassembly of focal adhesions, and increased apoptosis (40)—actions that are consistent with the increased apoptosis and tumor regression observed in the melphalan plus ADH-1 treatment groups. Like CrkL/Crk proteins, paxillin serves largely as an adaptor protein in focal adhesion complexes (27). pPaxillin is important in the regulation of focal adhesion assembly and associates with Crk/CrkL—important for cell motility (41, 42). Decreased phosphorylation of paxillin in response to ADH-1 could lead to both a loss of integrity of focal adhesion complexes and decreased association with Crk/CrkL, which could lead to increased apoptosis. Further studies will help clarify the link between N-cadherin disruption, increased CrkL/Crk phosphorylation, decreased paxillin phosphorylation, and why, in the presence of a DNA-damaging agent, such as melphalan, apoptosis and tumor regression increased so dramatically.

The observed changes in FGFR-1 expression after disruption of N-cadherin binding are consistent with previous reports (43), which showed that FGFR-1 stability at the membrane is dependent on its association with N-cadherin. These results hint that ADH-1 is disrupting extracellular N-cadherin adhesion, altering the association of N-cadherin and FGFR-1, and destabilizing FGFR-1. Potential downstream consequences of a loss of expression of FGFR-1 include altered signaling in the Ras g protein pathway, inhibition of epithelial-to-mesenchymal transition (44, 45), and decreased cell survival (46) consistent with the observed increases in cell death and tumor regression.

The serine/threonine protein kinase Akt, which is downstream of the PI3K signaling pathway, is an important cell survival protein and in its active or phosphorylated state (pAkt) can inhibit apoptosis. Our observation that pAkt increased in response to ADH-1 plus melphalan, although not consistent with the observed increase in apoptosis, may represent a compensatory mechanism in response to cell stress. Furthermore, it has been reported that high activity of Akt in breast cancer cell lines was not associated with decreased susceptibility to apoptosis nor did inhibition of Akt act to increase susceptibility (47).

Our results show for the first time the marked effectiveness of a novel therapeutic approach to melanoma treatment involving targeted therapy against the N-cadherin molecule to synergistically help improve both regional and systemic chemotherapy responses. ADH-1, a unique peptide designed to target N-cadherin adhesion complexes, shows minimal antitumor affects alone but dramatically improves tumor response in two different melanoma models using two different chemotherapies. ADH-1 treatment seems to make tumor cells more susceptible to apoptosis, which in the setting of regional therapy with melphalan is secondary to increased DNA adduct formation and activation of signaling pathways that may lower cellular threshold to apoptosis. As further studies focus on more clearly defining the mechanism behind the synergistic response, clinical phase I studies have already begun in both the regional and systemic settings with combination ADH-1 and chemotherapy. Given that the mean survival for patients with metastatic melanoma is only 6 to 9 months, new approaches such as targeting N-cadherin provide hope for improvement in current chemotherapeutic strategies.

Disclosure of Potential Conflicts of Interest

M. Gupta: Adherex Technologies, Inc. employee; W.P. Peters: Adherex Technologies, Inc. employee. D.S. Tyler and W.P. Peters are co-inventors of a pending patent titled "Cancer treatment methods using cadherin antagonists in combination with anticancer agents" (application number 60/848,624; 9/27/06); D.S. Tyler's rights to this patent have been signed over to the United States government. The other authors disclosed no potential conflicts of interest.

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