Targeting Nodal in Conjunction with Dacarbazine Induces Synergistic Anticancer Effects in Metastatic Melanoma

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Abstract

Metastatic melanoma is a highly aggressive skin cancer with a poor prognosis. Despite a complete response in fewer than 5% of patients, the chemotherapeutic agent dacarbazine (DTIC) remains the reference drug after almost 40 years. More recently, FDA-approved drugs have shown promise but patient outcome remains modest, predominantly due to drug resistance. As such, combinatorial targeting has received increased attention, and will advance with the identification of new molecular targets. One attractive target for improving melanoma therapy is the growth factor Nodal, whose normal expression is largely restricted to embryonic development, but is reactivated in metastatic melanoma. In this study, we sought to determine how Nodal-positive human melanoma cells respond to DTIC treatment and to ascertain whether targeting Nodal in combination with DTIC would be more effective than monotherapy. A single treatment with DTIC inhibited cell growth but did not induce apoptosis. Rather than reducing Nodal expression, DTIC increased the size of the Nodal-positive subpopulation, an observation coincident with increased cellular invasion. Importantly, clinical tissue specimens from patients with melanomas refractory to DTIC therapy stained positive for Nodal expression, both in pre- and post-DTIC tumors, underscoring the value of targeting Nodal. In vitro, anti-Nodal antibodies alone had some adverse effects on proliferation and apoptosis, but combining DTIC treatment with anti-Nodal antibodies decreased cell growth and increased apoptosis synergistically, at concentrations incapable of producing meaningful effects as monotherapy.

Implications: Targeting Nodal in combination with DTIC therapy holds promise for the treatment of metastatic melanoma. Mol Cancer Res; 1–11. ©2015 AACR.

Introduction

Metastatic melanoma is the leading cause of skin cancer deaths in the United States, with a median overall survival of only 6–9 months (1). Despite advances in the field, the reference therapy for patients diagnosed with metastatic melanoma is dacarbazine (DTIC), which was first approved for treatment in the 1970s. DTIC therapy is associated with poor patient outcome, with complete response occurring in less than 5% of cases (2). More recently, FDA approval has been granted for a limited number of alternative treatments, including vemurafenib (3) and dabrafenib (ref. 4; B-RAF inhibitors approved only for V600E B-RAF mutation-positive melanomas), trametinib (ref. 5; a MEK inhibitor also limited to B-RAF–mutant melanomas), as well as immunomodulating agents such as ipilimumab (ref. 6; an immunotherapeutic monoclonal antibody to CTLA-4), pembrolizumab (anti-PD1 monoclonal antibody; ref. 7), IFNo-1b (8), and IL2 (9). However, these monotherapies are typically restricted in their efficacy and tend to provide only a small improvement in progression-free survival before resistance develops (10, 11). As such, combinatorial targeting with multiple agents to overcome drug resistance has become an attractive potential strategy, but has so far had limited success (12–14). Thus, the identification of alternative targets and development of new therapies are still needed.

Nodal is a growth factor of the TGFβ superfamily. Predominantly expressed during early embryonic development, Nodal is important in embryonic stem cell maintenance and body axis establishment (15–17). Importantly, Nodal is not typically observed in most normal adult tissues but is reactivated in various advanced stage cancers such as metastatic melanoma, as well as breast, prostate, and ovarian carcinomas (18–23). Canonical Nodal signaling occurs via Nodal binding to type I (ALK4/7) and type II (ActRIB) activin-like kinase receptors in a Crypto-dependent or -independent fashion. Subsequent phosphorylation of Smad2/3/4 enables the complex to translocate to the nucleus and activate a transcriptional program that typically includes Nodal itself and the Nodal antagonist, Lefty (15–17). In advanced cancers, Nodal expression is maintained by a positive feedback loop (where signaling upregulates expression), but concurrent

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Lefty upregulation is absent due to DNA methylation of the promoter (19, 24), leading to unregulated Nodal expression and signaling.

Efforts to target Nodal in metastatic melanoma cells have shown inhibition of cell aggressiveness in vitro and reduced tumor growth in in vivo xenograft models (18, 19, 21, 25). It is not currently known how Nodal signaling is affected by standard therapy for metastatic melanoma nor whether targeting Nodal signaling offers any improvement over conventional monotherapy. In this study, we sought to identify the effects of DTIC on Nodal-expressing melanoma cell lines and evaluate the efficacy of targeting Nodal in combination with DTIC.

**Materials and Methods**

**Cells**

Melanoma cell lines utilized were: C8161 (University of Arizona, Tucson, AZ; 1999); MV3 (a gift of Dr. van Muijen, University Hospital Nijmegen, Nijmegen, the Netherlands; 2006); SK-MEL-28 (ATCC, 2010). Cell lines were authenticated by short tandem repeat genotyping at Lurie Children’s Hospital of Chicago (Chicago, IL; 2009–2010). Lines were routinely tested for mycoplasma contamination with a PCR ELISA Kit (Roche). All cell lines were maintained as previously described (21).

**Chemicals and antibodies**

Dacarbazine (DTIC) was dissolved in serum-free medium at stock concentrations before use (KLT Laboratories). Antibodies used were mouse anti-actin (Calbiochem), mouse anti-BCL2 (Santa Cruz Biotechnology), rabbit anti-phospho-HistoneH3 (Ser10), rabbit anti-HistoneH3, and rabbit anti-PARP (Cell Signaling Technology), rabbit anti-Nodal (dialyzed to remove preservative contamination prior to cell culture treatments; Santa Cruz Biotechnology), mouse anti-Nodal (Abcam, immunohistochemistry), donkey anti-rabbit AlexaFluor-488, and anti-mouse AlexaFluor-546 (Life Technologies).

**Drug and antibody treatments**

For DTIC experiments, working concentrations were prepared immediately before application. Control conditions employed serum-free medium alone that was diluted equivalent to the highest concentration of DTIC. Cells were typically evaluated after 72 hours. For immunofluorescence experiments, cells were seeded on glass coverslips. For antibody experiments, cells were antagonized with rabbit anti-Nodal antibodies or whole-molecule rabbit IgG (Jackson ImmunoResearch Laboratories) diluted in medium, and were analyzed after 72 hours. In combination experiments, parallel cultures were incubated in DTIC diluted to 5 μg/mL or equivalent volume of serum-free medium (control) for 72 hours, after which cells were washed and medium was replaced every 24 hours. After 72 hours, anti-Nodal antibodies or rabbit IgG diluted to 3 μg/mL was added to cells and cultured for 72 hours before analysis.

**Flow cytometry**

Cell viability assays were evaluated on a Guava EasyCyte HT Flow Cytometer using Guava viacount reagent according to the manufacturer’s instructions (Millipore). Parameters were set using untreated cells. Averages of triplicate samples were analyzed for each data point.

**Western blot analysis**

Whole-cell lysates were prepared as previously described (18), and SDS-PAGE gel electrophoresis and Western blotting used standard techniques. Polyvinylidene difluoride membranes were blocked in 5% nonfat milk, and antibodies diluted in either 5% milk or 5% bovine serum albumin overnight at 4°C, depending on the manufacturer’s recommendations. Signal was detected using West Pico Chemiluminescence Reagent (ThermoFisher). Membranes were stripped between antibodies using Restore Western blot stripping buffer (ThermoFisher). Specifically for Nodal, relative protein levels were evaluated using ImageJ software with actin as reference.

**Immunofluorescence**

For phospho-Histone H3 staining, cells on coverslips were fixed with 4% paraformaldehyde, and washed, blocked, and incubated in antibodies according to the manufacturer’s recommended protocol (Cell Signaling Technology). For Nodal staining, cells on coverslips were fixed with ice-cold methanol, washed, blocked, and incubated in antibodies as previously described (23). Staining was visualized on a Zeiss LSM510 Meta confocal microscope under a 25x LD.LCI.PlanApo multi-immersion objective and images captured using ZEN 2009 software (Zeiss). Cells were counted in at least 5 random fields in each of three independent experiments. Mean and SD was presented graphically.

**Reverse transcription and real-time PCR**

Messenger RNA was isolated from cells using the PerfectPure Cell RNA Isolation Kit (5Prime). Reverse transcription and real-time PCR (RT-PCR) were performed as previously described using TaqMan gene expression primer-probes (Applied Biosystems): Nodal (Hs00250630_s1) and RPLPO large ribosomal protein (4333761F). Experiments included cDNA samples generated with no reverse transcriptase to verify the absence of DNA contamination. Experiments were run in triplicate wells and were performed at least three times.

**Matrigel invasion assays**

Invasion assays were performed using BD biocoat growth factor-reduced Matrigel invasion chambers in accordance with the manufacturers’ protocol (Corning). Briefly, after treatment, cells were plated in triplicate and invaded cells adhered to the underside of the chamber were stained with 0.1% crystal violet. Dye was extracted using 10% acetic acid and optical density was measured on a Bio-Rad plate reader at 570 nm. Raw data were averaged and converted to percentage of control for graphing. For DTIC experiments, cells were treated with different DTIC concentrations for 72 hours as described, the medium removed and cells washed every 24 hours for 72 hours before plating. For combination experiments, cells were treated with DTIC followed by anti-Nodal antibodies, rabbit IgG, or medium only control as described before plating.

**Patient samples and immunohistochemistry**

Archival formalin-fixed and paraffin-embedded melanoma tissue sections from five patients that failed to respond to DTIC therapy were obtained from the Biospecimen Bank of the Melanoma Institute Australia. Immunohistochemistry was performed on a Microm HMS710i autostainer using mouse anti-Nodal (19). Color was developed with 3,3′-diaminobenzidine and sections
were counterstained with Hematoxylin (Biocare Medical). Adjacent serial sections were incubated with ChromPure mouse IgG (Jackson Immunoresearch Labs) as negative control. Sections were visualized and digital images captured and analyzed using a Leica DM4000B microscope.

Multicellular tumor spheroid (MCTS) assays

Spheroids were generated essentially as previously described (26). C8161 cells were diluted in complete medium or in medium containing DTIC (5 μg/mL) and either rabbit IgG or anti-Nodal antibodies (3 μg/mL). Each condition was replicated in 16 wells of a 96-well plate precoated with 1% (w/v) agar. Cells were force aggregated by centrifugation at 1,000 × g. Plates were incubated for 72 hours before analysis. Spheroids were fixed in 4% paraformaldehyde overnight at 4°C, before being dehydrated and embedded in paraffin wax. Parallel 4 μm sections were deparaffinized and rehydrated before staining. Hematoxylin and eosin staining used standard techniques. Immunofluorescence labeling of phospho-Histone H3 was performed using the recommended protocol except that antigen retrieval was performed in citrate buffer (pH 6.0) for 37 minutes at 97°C. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was performed using a kit according to the manufacturer's protocol (Millipore). Sections were visualized and images captured as described above, except that a × 10 LD.LCI.PlanApo multi-immersion objective was utilized. For clarity, phospho-Histone H3 fluorescent was pseudocolored red after image capture to distinguish it from TUNEL staining.

Results

Effects of DTIC on Nodal-expressing melanoma cells in culture

To address how exposure to DTIC affects Nodal-expressing, metastatic melanoma cells, we treated three cell lines (C8161, SK-MEL-28, and MV3) with increasing doses of DTIC for 72 hours and evaluated cells by flow cytometry. Compared with control cells that were exposed only to carrier, increasing doses of DTIC resulted in a reduction in total viable cells in the population that seemed to plateau at the highest concentrations tested (Fig. 1A). Importantly, the response to DTIC was somewhat dissimilar among cell lines; C8161 being the most sensitive cells, MV3 having an intermediate response, and SK-MEL-28 cells being least sensitive. Based upon this information, we identified the dose of DTIC responsible for a 50% reduction in cell population size (5 μg/mL for C8161, 10 μg/mL for MV3, and 25 μg/mL for SK-MEL-28) and the lowest dose capable of achieving an apparent maximal effect (25 μg/mL for C8161, 50 μg/mL for MV3, and 150 μg/mL for SK-MEL-28; Supplementary Fig. S1A–S1C). These identified concentrations were then utilized in subsequent experiments.

To determine whether the response of these cell lines to DTIC was a consequence of effects on cellular growth, survival, or a combination of the two, cells treated with DTIC were analyzed by Western blot analysis for markers of cell proliferation [phospho-Histone H3 (Ser10)] and apoptosis (cleaved PARP; Fig. 1B). In comparison with untreated controls, increasing doses of DTIC caused a dose-dependent reduction in Histone H3 phosphorylation, whereas total Histone H3 levels remained consistent across treatment groups. Conversely, PARP cleavage was not observed in either C8161 or SK-MEL-28 cells at any drug concentration, while in MV3 cells, some cleaved PARP was detected at the highest concentration tested. To ascertain the response of these cells over time, parallel treated wells were collected at 24-hour time points, and analyzed by flow cytometry for cell population size and programmed cell death. Compared with control cells that expanded in a fairly linear fashion and underwent 1 to 2 doublings in the 72-hour time frame, DTIC treatment caused a dose-dependent reduction in the expansion rate (Fig. 1C). To determine whether this response was due to cell death as well as growth inhibition, we also analyzed the proportion of dead cells and found little difference among the treatment groups (Fig. 1D), suggesting that the effect of DTIC is predominantly on restricting cellular growth. To evaluate this on a cell-by-cell basis, we performed immunofluorescence studies of phospho-Histone H3 on DTIC-treated cells (Fig. 1E). Compared with control cells where numerous nuclei contained phosphorylated Histone H3, DTIC treatment caused a dose-dependent reduction of phospho-Histone H3–positive nuclei. Of note, positive cells were rarely detected at the highest concentrations. In general, the difference in phospho-Histone H3–positive nuclei between control and treated cells was statistically significant (Supplementary Fig. S1D). Collectively, this suggests that DTIC treatment limits population growth predominantly by reducing cell proliferation and not by inducing apoptosis.

Residual cells remaining after DTIC treatment maintain expression of Nodal and are more invasive than parental cells

Nodal expression in cells treated with increasing doses of DTIC was evaluated at the mRNA level by reverse transcriptase real-time PCR (RT-PCR) and at the protein level by Western blot analysis. Nodal mRNA levels were relatively unchanged among treatment groups (Fig. 2A) and Nodal protein levels were unchanged or slightly elevated relative to untreated cells (Fig. 2B). Given that we have previously described Nodal protein to be limited to a subpopulation of cells in culture (21), we also analyzed Nodal protein by immunofluorescence (Fig. 2C and Supplementary Fig. S2A and S2B). Surprisingly, compared with untreated control cells that displayed the typical proportion of Nodal–positive cells (Fig. 2D; 22–31%), cultures exposed to increasing doses of DTIC contained a greater percentage of Nodal–positive cells that typically increased proportionally with higher dosages of DTIC. In all cases, this was significantly different from controls (P < 0.05). Taken together, this suggests that residual cells persisting after DTIC exposure retain Nodal expression.

To determine whether the residual cells surviving DTIC treatment differed from parental cells in their aggressiveness, we evaluated invasive behavior by exposing C8161 and SK-MEL-28 cells to DTIC before plating in Matrigel invasion assays. Compared with controls, cells treated with DTIC typically exhibited a dose-dependent increase in the proportion of invaded cells (Fig. 2E). This increase was significantly greater than control cells at the highest concentrations tested (P < 0.05), suggesting that DTIC-resistant residual cells exhibit more invasiveness, an observation coincident with the increase in Nodal-positive cells.

DTIC therapy fails to eliminate Nodal-positive cells from patient melanomas

To determine how Nodal expression is affected in human melanomas treated with DTIC, biopsy samples from a cohort of five relapsed patients whose melanomas were refractory to DTIC therapy were evaluated for Nodal. Tissue sections from tumors before and after DTIC therapy were labeled with anti-Nodal

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Synergistic Effect of DTIC and Nodal Targeting in Melanoma
A

Total viable cells in population (as % relative to untreated control)

DTIC concentration (μg/mL)

B

DTIC concentration (μg/mL)

pHistoneH3
HistoneH3
full-length PARP
cleaved PARP
Actin

C8161
MV3
SK-MEL-28

C

Viable cells (% of plated)

DTIC concentration (μg/mL)

0 5 25 0 5 10 50 0 5 25 150

C8161 MV3 SK-MEL-28

D

% Cell death

DTIC concentration (μg/mL)

0 5 25 10

C8161 MV3 SK-MEL-28

E

Phospho-HistoneH3

DAPI

No DTIC

5 μg/mL

25 μg/mL

No DTIC

5 μg/mL

25 μg/mL

No DTIC

5 μg/mL

25 μg/mL

No DTIC

5 μg/mL

25 μg/mL

No DTIC

5 μg/mL

25 μg/mL
Antibodies and expression determined by immunohistochemistry (Fig. 3). In all patients, Nodal expression was observed both in the specimens collected before exposure to a DTIC treatment regime and in biopsy tissue taken following DTIC therapy. The observation of Nodal protein in both DTIC-naive and residual DTIC-resistant melanomas offers proof-of-concept support for Nodal as a potential drug target in combination with chemotherapy.

**Anti-Nodal antibodies adversely affect proliferation and apoptosis at high concentrations**

Considering that Nodal expression is maintained in cells exposed to DTIC, we reasoned that additional targeting of Nodal might improve the response of cells to DTIC treatment. Anti-Nodal antibodies have previously been utilized to inhibit Nodal signaling (23, 25), but have not been extensively evaluated in melanoma cells. To first determine how cultured cells respond to Nodal inhibition (independent of DTIC treatment), we exposed cells to increasing concentrations of anti-Nodal antibodies for 72 hours, and evaluated cell growth and death by flow cytometry. Compared with untreated or rabbit IgG-treated control cells, cultures of C8161 and SK-MEL-28 cells treated with anti-Nodal antibodies at intermediate (3 μg/mL) and high (4–5 μg/mL) concentrations exhibited a reduced viable cell population size, that was significantly different from controls at the highest concentrations (Fig. 4A and Supplementary Fig. S3A; P < 0.05).

Furthermore, cells exposed to high concentrations of anti-Nodal antibodies (4–5 μg/mL) exhibited increased cell death that was significantly different from control cells (Fig. 4B and Supplementary Fig. S3B; P < 0.05). In C8161 cells, the majority of the population remained viable (54%), even at the highest antibody concentration evaluated, while, in SK-MEL-28 cells, death of the majority of cells (78%) was observed at the highest concentration.

To confirm the effects of anti-Nodal antibodies, Nodal protein levels, as well as markers of proliferation and apoptosis, were evaluated in cell lysates (Fig. 4C). Importantly, Nodal expression was downregulated in a concentration-dependent manner verifying that Nodal signaling was decreased by anti-Nodal antibodies (expected as Nodal expression is maintained by a positive feedback loop). Similarly, a decrease in Histone H3 phosphorylation was observed with increasing concentrations of anti-Nodal antibodies, while total Histone H3 levels remained constant. Surprisingly, when we evaluated apoptosis, we were unable to detect PARP cleavage, even at the highest concentrations of antibodies tested. As we had observed an increase in cell death by flow cytometry, we evaluated levels of an alternative apoptosis marker, B-cell lymphoma 2 (BCL2; an antiapoptotic protein). In contrast to lack of PARP cleavage, we did observe a decrease in BCL2, suggesting the likely early induction of apoptosis.

**Targeting Nodal in addition to DTIC treatment is significantly more effective than DTIC alone**

To determine how cells would respond to anti-Nodal antibodies in addition to DTIC treatment, we pretreated cultures with DTIC, washed the cells, and then exposed cultures to anti-Nodal antibodies. At the lowest tested DTIC dose of 5 μg/mL, we observed that further exposing cells to anti-Nodal antibodies at an intermediate concentration (3 μg/mL; only modestly effective by itself; Fig. 4 and Supplementary Fig. S3) was sufficient to induce a dramatic effect on cells. In contrast to all other treatment groups, cells that were pretreated with DTIC followed by anti-Nodal antibodies exhibited a striking decrease in the viable cell population (Fig. 5A and Supplementary Fig. S4A; P < 0.05). In contrast, cells exposed to DTIC and anti-Nodal antibodies displayed a striking increase in the proportion of programmed cell death that was significantly greater than untreated cells or cells exposed only to antibody (Fig. 5B and Supplementary Fig. S4B; P < 0.05).

Importantly, the DTIC and anti-Nodal antibody combination affected the majority of the cell population (78% in C8161 cells; Fig. 5B, 75% in SK-MEL-28 cells; Supplementary Fig. S4B). We observed a concurrent significant decrease in Nodal mRNA levels in C8161 cells exposed to DTIC and anti-Nodal antibodies (P < 0.05; Fig. 5C), as well as a dramatic decrease in Nodal protein, a complete loss of Histone H3 phosphorylation, and an increase in PARP cleavage, that was not observed under any other conditions (Fig. 5D). Taken together, these data suggest that combining DTIC and anti-Nodal antibody treatments has a synergistic effect on apoptosis, far greater than either single treatment at the corresponding concentrations.

To address how combined DTIC and anti-Nodal treatments might affect cell behavior, invasiveness was evaluated in SK-MEL-28 cells treated with DTIC alone, or with DTIC followed by rabbit IgG or anti-Nodal antibodies. After treatment, cells were plated in Matrigel invasion assays where cultures treated with DTIC and anti-Nodal antibodies exhibited a significant reduction in cellular invasion compared with DTIC control (Fig. 5E; P < 0.05). These data suggest that targeting Nodal impairs the invasive ability of DTIC-resistant melanoma cells.

**Cells grown in multicellular tumor spheroid culture also respond to combinatorial DTIC and anti-Nodal antibody treatment by undergoing apoptosis**

The multicellular tumor spheroid (MCTS) assay more accurately represents *in vivo* tumors than monolayer cell cultures given its three-dimensional (3D) cellular arrangement (27). To address the response of C8161 cells grown in 3D culture to combined DTIC and anti-Nodal antibody exposure, MCTS cultures were formed by centrifugation and grown either untreated, with DTIC and rabbit IgG as a control, or with DTIC and anti-Nodal antibodies (Fig. 6). Spheroids were fixed and serial sections were analyzed for proliferation and apoptosis using phospho-Histone H3 staining and TUNEL assay, respectively. While some phospho-Histone H3–positive cells were detected in MCTS sections from untreated and control cells, positive cells were rarely detected in sections of MCTS samples treated with DTIC and anti-Nodal antibodies (compare Fig. 6C with Fig. 6A and B). In contrast,
Nodal mRNA expression (% relative to untreated control)

DTIC concentration (µg/mL)

No DTIC  0  5  10  25  50  100  150  200

40  60  80  100  120  140  160  180  200

C8161  MV3  SK-MEL-28

Nodal expression:

Actin expression:

DAPI Nodal

DAPI Nodal Actin

% Nodal-positive cells

Cell invasion (as % relative to no DTIC control)

C8161

No DTIC  5 µg/mL DTIC  25 µg/mL DTIC

SK-MEL-28

No DTIC  5 µg/mL DTIC  25 µg/mL DTIC  150 µg/mL DTIC

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Synergistic Effect of DTIC and Nodal Targeting in Melanoma

TUNEL-positive, apoptotic cells were uncommon in untreated and control MCTS sections, but were abundant in MCTSs exposed to DTIC and anti-Nodal antibodies (compare Fig. 6C with Fig. 6A and B). This suggests that combinatorial targeting also limits proliferation and induces apoptosis in a 3D assay.

Nodal expression is maintained and targetable in V600E B-RAF mutation-positive melanoma cells surviving vemurafenib treatment

While DTIC is considered an older conventional therapy, targeted therapies, such as B-RAF inhibitors, are among newer strategies aimed at improving patient response. Therefore, we performed initial experiments to determine whether the persistence of Nodal is unique to cells surviving DTIC or is common to cells acquiring resistance to other drug treatments. SK-MEL-28 cells (harboring the V600E B-RAF mutation) were treated with increasing doses of the B-RAF inhibitor RG7204 (vemurafenib) for 72 hours. Compared with DMSO-treated control cells, RG7204 treatment caused a dose-dependent reduction in the number of viable cells that plateaued at around 0.5 μmol/L, leaving approximately 50% of the cell population intact (Supplementary Fig. S5A). Importantly, in cell lysates, Nodal protein was detected at similar levels at all RG7204 concentrations tested (0.1–10 μmol/L; Supplementary Fig. S5B). To verify potency, ERK activity was evaluated in lysates from parallel cultures treated with RG7204 for 1 hour. A dose-dependent decrease in phosphorylated ERK1/2 was observed compared with total ERK1/2 (data not shown). In contrast, a Nodal-positive, B-RAF wild-type cell line (C8161) failed to respond to RG7204 at any concentration tested (0.1–10 μmol/L) and ERK phosphorylation was not altered (data not shown).

To evaluate how treatment with anti-Nodal antibodies affects SK-MEL-28 cells surviving RG7204 treatment, cultures were first treated with 0.5 μmol/L RG7204, then surviving cells exposed to anti-Nodal antibodies. Compared with cells pretreated with RG7204 (RG7204-resistant cells) or with RG7204-resistant cells exposed to rabbit IgG that doubled twice over 72 hours, RG7204-resistant cultures exposed to anti-Nodal antibodies failed to expand (Supplementary Fig. S5C). Coincident with this, anti-Nodal antibody-treated cells exhibited a marked decrease in viability (Supplementary Fig. S5D), and dramatic increase in cell death (Supplementary Fig. S5E) that was significantly different from control ($P < 0.05$). Taken altogether, these data indicate that Nodal expression maintained in B-RAF inhibitor–resistant cells is also targetable with anti-Nodal antibodies. While this line of inquiry is not considered the primary focus of our study, the results are complementary and highly informative regarding the persistence of Nodal-positive subpopulations that are unaffected by B-RAF–targeted therapy.

Discussion

Despite recent advances in the field, metastatic melanoma is still the leading cause of skin cancer deaths and remains highly refractory to current treatment strategies. In addition to the historically poor patient response to DTIC therapy, more recently FDA-approved treatments are unsuitable for many patients and tumors typically develop resistance to these agents over time (10, 11). The concept of combinatorial targeting is an attractive means of overcoming drug resistance in melanoma. Previous preclinical and clinical studies have addressed possible ways to circumvent the poor response to DTIC by including additional chemotherapeutic agents (12) or by combining DTIC with targeted molecular inhibitors (28–33). In this study, we are first to establish Nodal as a rational combinatorial target in melanoma and present a novel, synergistic combination of DTIC and anti-Nodal antibodies. The reexpression of Nodal in metastatic melanoma and other aggressive cancers is associated with advanced disease progression, and contributes to the aggressive behavior of cancer cells in vitro and in vivo (18, 19, 21, 23, 25). Hence, our
preclinical study targeting Nodal in combination with an already standardized treatment is a potentially exciting therapeutic strategy that warrants future indepth investigation. In this study, in agreement with others, we show that DTIC treatment limits cell growth, but does not induce apoptosis in a variety of melanoma cell lines (29, 30). Of note, these cell lines differ in their B-RAF status: C8161 and MV3 cells are B-RAF wild-type, while SK-MEL-28 cells carry the V600E B-RAF mutation. While we observed SK-MEL-28 cells to be the most resistant to DTIC treatment and C8161 cells to be the most sensitive (Fig. 1), the B-RAF status of these cells likely does not drastically impact resistance to DTIC as, in our hands, combining DTIC with the B-RAF inhibitor RG7204 has a minimal effect over either single agent (K. Hardy and M. Hendrix, personal communication). The three metastatic melanoma cell lines (C8161, MV3, and SK-MEL-28) were primarily chosen for study because they have previously been shown to express Nodal (18, 21) and not because of their B-RAF status, thus indicating that Nodal expression in melanoma cells does not appear to correlate with B-RAF status. It is clear from our study that, regardless of the B-RAF status, Nodal continues to be expressed in these cells following DTIC treatment, even at high drug concentrations. Complementary to this, Nodal was also observed to persist in tissue samples from melanomas refractory to DTIC therapy. While the B-RAF status of these tumors is not known, as approximately 50% of all melanomas are thought to harbor a B-RAF mutation (34), it is possible that at least one patient had B-RAF mutation-positive melanoma. Also importantly, Nodal is still detected in B-RAF mutant SK-MEL-28 cells with acquired resistance to the B-RAF inhibitor, RG7204. Together, this suggests Nodal signaling as a possible broad combinatorial target to help circumvent drug resistance in both B-RAF wild-type and B-RAF mutation-carrying melanomas.

Certainly in B-RAF mutation-positive melanomas, the MAPK pathway is a critical effector of tumor progression. In patients treated with B-RAF and MEK inhibitors, resistance often develops via the reactivation of MAPK signaling (10). Importantly, drug resistance still develops with recently FDA-approved combined B-RAF/MEK inhibitor therapy (35, 36), likely via alternate MAPK pathway mutations (37). As targeting B-RAF mutations in melanoma does not eliminate MAPK dependency, the continued search for alternative targetable MAPK-inducing mechanisms is justified. In breast cancer, a noncanonical Nodal signaling pathway converges on MAPK signaling, and contributes to epithelial-mesenchymal transition, invasion, and tumor progression (38, 39). While it is not currently known whether MAPK signaling is activated downstream of Nodal in melanoma, given our observation that B-RAF inhibitor-resistant cells retain Nodal expression and are sensitive to Nodal inhibition, it is exciting to speculate that Nodal signaling may be an alternative mechanism capable of inducing MAPK signaling in melanoma that could be targeted in combination with other MAPK-directed therapies.

Tumor cell heterogeneity is now a well-accepted phenomenon in vitro and in vivo (40, 41). Intracellular Nodal protein was previously detected in only a subpopulation of melanoma cells in culture (21), highlighting its heterogeneous expression pattern. In agreement with this, we observe Nodal protein in 22%–31% of untreated cells (depending on the cell line) in the current study (Fig. 2). Interestingly though, in cell lines treated with DTIC, we detect a dose-dependent increase in the proportion of Nodal-positive cells by immunofluorescence. One possible explanation for this observation is that Nodal-expressing cells are somehow insensitive to DTIC treatment. Another possibility is that Nodal expression is upregulated by DTIC. The latter is unlikely as Nodal mRNA levels remain relatively unchanged by all DTIC concentrations, suggesting no impact on Nodal gene transcription.
Surprisingly, relative Nodal protein levels in cell lysates from DTIC-treated cells remain either unchanged or only slightly elevated compared with untreated control cells, suggesting no significant quantitative difference on a cell population basis, despite the observed expanded Nodal-positive subpopulation. The reasons for this discrepancy are unclear, but the expansion of the Nodal-positive subpopulation is a likely explanation for the dramatic effect on cells in combinatorial DTIC and anti-Nodal antibody experiments. We show that combinatorial treatment renders cells significantly more sensitive to apoptosis than either single treatment, resulting in the death of close to 80% of cells in culture (Fig. 5). Importantly, at the concentration utilized in combinatorial experiments, DTIC treatment alone causes an expansion of the Nodal-positive subpopulation to approximately 60%, which could not fully explain the increased sensitivity to anti-Nodal antibodies. Thus, we reason that the disproportionate increase in cell death may be attributable to the “bystander effect”, whereby signals generated by one dying cell influence the survival of neighboring cells in a paracrine manner.

Despite the acceptance that tumor cell populations are heterogeneous with distinct subpopulations of cells that may retain the plasticity to transition between molecular phenotypes, the concept of a true stem cell population in cancer remains controversial (40, 41). Nodal signaling plays an important role in maintaining pluripotency in embryonic stem cells (43, 44), so it is conceivable that Nodal may also contribute to a cancer stem cell phenotype. However, as Nodal is a secreted protein, it has been challenging to specifically isolate and study the live Nodal-positive subpopulation to evaluate this possibility, as has been done with putative cancer stem cell surface markers (45, 46). Certainly, Nodal is important in tumor cell plasticity, as it is associated with vasculoenic mimicry in melanoma (18, 25, 47); specifically, down-regulation of Nodal suppresses this endothelial phenotype and abrogates vasculoenic mimicry (18, 25). In addition, the

Figure 5. Combining DTIC and anti-Nodal antibody treatment induces dramatic cell death. C8161 cells were either untreated or treated with 5 μg/mL DTIC, followed by no treatment, or 5 μg/mL anti-Nodal antibodies or rabbit IgG. Endpoint viable cell number graphed as a percentage of untreated cells (A) and percent cell death (B) were evaluated by flow cytometry. Nodal mRNA levels were evaluated using real-time RT-PCR (C). Protein lysates were analyzed by Western blotting for Nodal, Histone H3 phosphorylation at Ser10 (proliferation) and PARP cleavage (apoptosis). Actin was the loading control. #, significant difference from untreated cells and from cells treated only with anti-Nodal antibodies (P < 0.05). E, cellular invasion was evaluated in SK-MEL-28 cells pretreated with DTIC, then exposed to rabbit IgG or anti-Nodal or not further treated (no antibody) using Matrigel invasion chambers. Invading cells were quantified as percentage of no antibody control. #, P < 0.05.
heterogeneous cancer cell phenotype within a tumor may be associated with drug resistance, whereby cells with specific properties enabling them to withstand drug treatment are preferentially selected (48). Whether Nodal is specifically associated or simply coincident with a drug-resistant subpopulation is not entirely clear from the current study. Interestingly, a recent study of ABCB5-expressing melanoma cells (an ABC transporter associated with drug resistance) identified the selective survival of this subpopulation in response to DTIC treatment (49). Given that we observed a similar increase in the Nodal-positive subpopulation in the current study, it is exciting to speculate that Nodal may be associated with a drug-resistant subpopulation with plastic properties.

We found a synergistic increase in cell killing utilizing DTIC and anti-Nodal antibody concentrations incapable of producing meaningful effects alone, in cultured cells and tumor spheroids. This has potential significance for future in vivo studies where the 3D architecture, host immune response, and other side effects must be considered. DTIC at currently administered clinical dosages causes numerous side effects (12). Our study suggests the possibility that, in combination with anti-Nodal antibodies, the effective concentration of DTIC could be decreased, potentially reducing side effects. The efficacy of anti-Nodal antibodies has previously been tested in an experimental lung colonization nude mouse model using one metastatic melanoma cell line (25). This study showed both a decrease in lung melanoma colonies and an increase in apoptotic cells, suggesting that anti-Nodal antibodies can be effective in vivo models. Most noteworthy, the antibody concentration employed in the in vivo study was greater than that utilized in combinatorial experiments in the current in vitro study. While we understand that concentrations utilized in vitro are not necessarily directly applicable in vivo, the current study at least suggests the exciting possibility that combinatorial experiments may be effective. Thus, we conclude that the pursuit of in vivo combinatorial studies with DTIC and Nodal as a target are both warranted and should include the evaluation of agents at reduced concentrations that may be ineffective administered individually.

Disclosure of Potential Conflicts of Interest
M.J.C. Hendrix holds a patent to target Nodal. No potential conflicts of interest were disclosed by the other authors.

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