Cancer Therapy: Preclinical

An Antibody–Drug Conjugate Targeting the Endothelin B Receptor for the Treatment of Melanoma

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Abstract

Purpose: To identify and evaluate targets amenable to antibody therapy in melanoma.

Experimental Design: We searched for mRNA transcripts coding for cell-surface proteins with expression patterns similar to that of the melanoma oncogene MITF. One such candidate, the endothelin B receptor (EDNBR), was first analyzed for a functional contribution to tumor growth by conditional induction of shRNA. Second, antibodies were raised to the receptor, conjugated with monomethyl auristatin E, and tested for efficacy against melanoma tumor models generated from cell lines.

Results: Conditional knockdown of the receptor in tumor xenograft models resulted in only a modest impact on tumor growth. A monoclonal antibody reactive with the N-terminal tail of EDNBR was found to internalize rapidly into melanoma cells. When conjugated with monomethyl auristatin E, the antibody–drug conjugate (ADC) showed remarkable efficacy against human melanoma cell lines and xenograft tumor models that was commensurate with levels of receptor expression. Comparative immunohistochemistry revealed a range of EDNBR expression across a panel of human melanomas, with the majority expressing levels equivalent to or greater than that in the models responsive to the ADC.

Conclusion: An ADC targeting the EDNBR is highly efficacious in preclinical models of melanoma.

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Introduction

Melanoma is an aggressive form of skin cancer that has recently undergone an alarming increase in incidence (1). Although cures can be achieved with surgical resection of localized lesions, the advanced stages of melanoma are only poorly responsive to currently approved therapies. The 5-year survival rate for stage IV metastatic melanoma is approximately 10% (1). New therapeutic approaches, including antisense to Bcl2, antibodies to CTLA4, small molecule RAF kinase inhibitors, and adoptive immunotherapy, are currently in clinical testing for metastatic melanoma (2). The results from some of these recent studies seem to be encouraging, but a durable impact on overall survival will likely require therapeutic combinations including additional new agents.

More than 20 years ago, endothelin-1 (ET-1) was isolated from aortic endothelial cells and found to have potent vasoconstrictive activity (3). The receptors for endothelins were cloned shortly thereafter (4, 5) and their expression in various cell types, including melanocytes and melanoma cells, pointed to functions independent of their role in endothelium. It is now well recognized that the endothelin B receptor (EDNBR) is critical for the faithful derivation of melanocytic cells emanating from the neural crest during embryonic development (6, 7). Melanocyte precursors rely on EDNBR activity to proliferate and migrate from the neural tube to their final destinations (8, 9). Mice with defective genes coding for either EDNBR or endothelin-3 (ET-3) exhibit a pigmentation deficit in their coats and a shortage of enteric ganglion cells, also derived from the neural crest. These characteristics strongly resemble those associated with the WS4 variant of Waardenburg syndrome in humans, which has been attributed to germline mutations in either ET-3 or EDNBR (10–12). An additional variant of this syndrome, WS2, has been mapped to heritable mutations in the microphthalmia-associated transcription factor (MITF), a key regulator of melanocyte development and a melanoma proto-oncogene (13–15).

The strong genetic evidence linking EDNBR activity to the fate of melanoblasts underscores a potential role for this receptor in the progression of melanoma. The expression of EDNBR mRNA and protein was reported to increase during disease progression from dysplastic nevi to metastatic melanoma (16). Blockade of EDNBR activity by 2 independent small molecule inhibitors interfered with growth and survival of melanoma cells and tumor xenografts (17–19). These preclinical studies implicate EDNBR as a potential driver of melanoma progression. However,
translational aspect of EDNBR inhibition by small molecules into the melanoma clinic has been somewhat disappointing. No objective responses were observed in phase II trials in which bosentan, a dual endothelin A/B-receptor antagonist, was administered either as monotherapy or in combination with dacarbazine in first-line therapy (20, 21).

To identify targets suitable for antibody therapy in melanoma, we carried out a search for transcripts encoding cell-surface proteins with expression correlative to that of MITF, the melanoma oncogene. Accordingly, EDNBR was readily identified in a panel of metastatic melanomas and melanoma cell lines. Herein, we describe the application of antibodies to EDNBR and, in particular, report that EDNBR is an extremely efficient target for antibody–drug conjugate (ADC) therapy.

Materials and Methods

EDNRA and EDNBR cDNAs

Human EDNRA cDNA from OriGene, human EDNBR cDNA from Incyte, mouse EDNBR cDNA from Image (Invitrogen), cynomologus monkey EDNBR cDNA from liver and lung cDNA libraries, and rat EDNBR cDNA from neonatal cDNA library were cloned into a CMV promoter-driven N-terminal FLAG-tagged vector. These constructs were used to generate Δ64 EDNBR lacking the N-terminal 64 amino acids and the EDNRA/EDNBR chimeras in which one of each of the 4 extracellular domains of EDNRA was substituted into EDNBR. The N-His-ΔSP-EDNBR cDNA coding for N-terminal 8xHis-tagged EDNBR protein without native signal peptide was subcloned into Baculovirus vector pAcGP67-B (BD Biosciences) and employed for the production of immunogen used to raise the 5E9 antibody.

Immunologic procedures

For production of monoclonal antibodies (mAb), BALB/c mice were immunized with either HEK293 cells stably transfected with full-length EDNBR clone or purified EDNBR protein. Immunization, hybridoma selection, and antibody purification were done as described previously (22).

For fluorescence-activated cell sorting (FACS), cells were harvested in PBS with 2.5 mmol/L EDTA and washed in PBS buffer containing 1% FBS. All subsequent steps were carried out at 4°C. Cells were incubated for 1 hour each with 2 to 5 μg/mL primary antibodies, followed by the appropriate secondary antibodies. Cells were then analyzed with a FACS Calibur flow cytometer (BD Biosciences). Primary antibodies, monoclonal or chimeric in-house generated anti-EDNBR antibodies (5E9 and 24C7) for EDNBR cell surface detection, FLAG M2 mAb (F-3165; Sigma Life Science) for N-Term FLAG tag detection were used. Alexa 488–conjugated anti-mouse or anti-human IgG fluorescent detection reagent (A11017, A11013; Invitrogen) was used.

For Western blotting, the following reagents were used for detection of proteins: anti-EDNBR in-house generated mAb 1H1.8.5, rabbit polyclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (PA1-987; Affinity Bioreagents), p44/42 mitogen-activated protein kinase (MAPK) and phospho-p44/42 MAPK [(Thr202/Tyr 204)] 9102, 9101S; Cell Signaling Technology], and β-tubulin (556321; BD Pharmingen/BD Biosciences).

Detection reagents Alexa 680–conjugated anti-mouse or anti-rabbit IgG (A21057, A21076; Molecular Probes/Invitrogen) and IRDye800-conjugated anti-mouse or anti-rabbit IgG (610-132-121, 611-132-122; Rockland Immunocorehematics for Research) were used.

For immunofluorescence, 10,000 cells were plated on 4-well chamber slides and incubated for 72 hours at 37°C in 5% CO2. Cells were fixed with 4% Ultra Pure Formaldehyde (18814; Polysciences Inc.) and permeabilized with 0.05% saponin (S-4521; Sigma Life Science) for N-Term FLAG tag detection were used. Alexa 488–conjugated anti-mouse or anti-human IgG fluorescent detection reagent (A11017, A11013; Invitrogen) was used.

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EDNBR immunohistochemistry on tissue sections was carried out on the Ventana Discovery XT Platform, using CC1 standard antigen retrieval as a pretreatment. Sections were incubated with goat anti-human EDNBR primary antibody (sc-21196; Santa Cruz Biotechnologies) at 0.05 μg/mL (1:4,000) for 60 minutes at 37°C, followed by incubation with an unconjugated rabbit anti-goat IgG secondary antibody (Vector Labs) at 5 μg/mL (1:200) for 32 minutes at room temperature. Sections were subsequently incubated for 16 minutes with anti-rabbit Omni-Map HRP reagent (Ventana Medical Systems), followed by Ventana DAB colorimetric peroxidase substrate. Sections were counterstained with Ventana Hematoxylin II reagent and subsequently dehydrated, cleared with xylenes, and coverslipped. ChromPure goat IgG (005-000-003; Jackson ImmunoResearch) was used as the isotype control. The human melanoma tissue samples were purchased from US Biomax, Inc. The overall staining intensity of tissue samples was scored as none (0), weak (1+), moderate (2+), or strong (3+).

Anti-EDNBR binding sites per cell was determined by Scatchard analysis essentially as described previously (22).

**Cell culture**

The cell lines A2058, A375, C32, COLO 829, G-361, Hs-294-T, Hs-695-T, LOX-IMVI, Malme-3M, MDA-MB-435, MeWo, RPMI-7951, SK23, SK-MEL-23, SK-MEL-28, UACC-257, and WM-266-4 were obtained from the American Type Culture Collection or NCI-60 [National Cancer Institute (NCI)] and the cell lines 526 mel, 537 mel, 624 mel, 888 mel, 928 mel, and 1,300 mel were a generous gift from Paul Robbins (Center for Cancer Research, Tumor Immunology Section, NCI) and were grown in appropriate media at 37°C and 5% CO2. Short hairpin RNA (shRNA)-mediated knockdown was induced in clonal populations by culturing these cells for 72 hours in complete medium containing 2 μg/mL doxycycline (631311; Clontech Laboratories). shRNA-bearing clonal populations were selected by the addition of 1 to 5 μg/mL Geneticin (10131-027; Gibco/Invitrogen). Cells were transfected with DNA, using Lipofectamine 2000 (11668-019; Invitrogen) as per the recommended protocol. To assess the effects of ADCs on cell viability, cells were plated at 1,500 per well in 50 μL of normal growth medium in 96-well clear-bottom plates. Twenty-four hours later, an additional 50 μL of culture medium with serial dilutions of antibodies was added to triplicate wells. Five days later, cell survival was determined using CellTitre-Glo Luminescent Cell Viability Reagent (G7572; Promega Corporation) and with an EnVision 2101 Multilabel Reader (Perkin-Elmer).

Cell lines with inducible shRNA-bearing lentivirus constructs were generated essentially as described previously (23–25). shRNA targeting EDNBR at nucleotides 1,363 to 1,381 of NM_000115, target sequence 3’-GAATTCCTGCATTAGAAGAA-5’ (D-003657-02; Dharmacon RNAi Technologies), was used to knockdown EDNBR in melanoma cell lines. The experiments were controlled using shRNA targeting luciferase gene. Mutant EDNBR gene, impervious to knockdown by this shRNA, was generated by changing target nucleotide sequence to 5’-AGCTTAAA-CAGTGTATCA-3’ while maintaining the protein identity.

**MAPK phosphorylation and calcium influx assays**

For MAPK phosphorylation, 2 × 10⁶ UACC-257 cells were serum starved for 24 hours on a 60-mm dish. Antibody 24C7 (5 μg/mL) or BQ788 sodium salt (100 nmol/L; PED-3788-PI; Peptide International Inc.) was added and the cells were incubated for 1 hour at 37°C prior to the addition of 100 nmol/L human ET-1 (PED-4198-v; Peptide International Inc.) for 5 minutes at 37°C. Cell lysates were probed for total or phosphorylated p44/42 MAPK and for β-tubulin. Protein bands were quantitated using Odyssey infrared imaging system from LI-COR Biosciences. Phosphorylated p44/42 (Thr202/Tyr204) MAPK bands were normalized to total p44/42 MAPK and the β-tubulin loading control.

For calcium influx, 25,000 HEK293 cells transfected with EDNBR were plated on a 96-well black-sided plate and incubated at 37°C, 5% CO2. After 48 hours, medium was aspirated and cells were loaded with dye for 2 hours at 37°C, using FLIPR Calcium 3 Assay Kit (catalogue no. R8090; Molecular Devices). Loading dye was spiked with 5 μg/mL 24C7 Ab or 100 nmol/L BQ788 sodium salt as appropriate, prior to addition to cells. The plate was loaded onto the FLIPR High-throughput Cellular Screening System (Molecular Devices) to obtain instant readout of the calcium mobilization on addition of 100 nmol/L ET-1.

**Xenograft models**

All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Ref: Institute of Laboratory Animal Resources (NIH publication no. 85-23), Washington, DC: National Academies Press; 1996).

Antibodies were conjugated with monomethyl auristatin E (MMAE) as described previously (26). The drug to antibody ratio for 5E9-vc-MMAE ranged from 2.95 to 3.55, and the drug to antibody ratio for control IgG-vc-MMAE ranged from 2.5 to 3.2. Average tumor volumes with SDs were determined from 10 animals per group. For efficacy studies with ADCs, 6- to 8-week-old female CRL Nu/Nu mice from Charles River Laboratories were inoculated subcutaneously in the dorsal right flank with 10 × 10⁶ UIACC-257 or with 5 × 10⁶ A2058 cells in HBSS with Matrigel. When tumor volumes reached approximately 200 mm³ (day 0), animals were randomized into groups of 10 each and administered a single intravenous (IV) injection of anti-EDNBR chimeric or mAb conjugated to MMAE through the valine-citrulline linker (26). MMAE conjugated to anti-gD antibody (human isotype) or to anti-ragweed antibody (mouse isotype) was used as a control. Tumor volumes were measured twice per week until study end. The same procedure was followed for growth studies involving A2058 and UIACC-257 cell lines expressing inducible shRNA, except that when tumor volumes reached approximately 200 mm³...
(day 0), animals were randomized and treated with either 5% sucrose water or 1 mg/mL doxycycline in 5% sucrose water. All water bottles were changed thrice weekly.

**Protein purification**

For the purification of ligand-bound His-tagged EDNBR protein, we followed a procedure involving expression in insect sf9 cells essentially as described previously (27), with a modification to the extraction of cell membranes. Protein was solubilized by adding 1% n-dodecyl-β-D-maltopyranoside (DDM) and 15% glycerol and nutating the mixture overnight at 4°C. Supernatant was collected after high-speed centrifugation and adjusted to 300 mmol/L sodium chloride concentration and pH 8.0. The sample was loaded on to Ni-NTA Superflow Column (30410; Qiagen). The column was washed with PBS buffer adjusted to 300 mmol/L sodium chloride, 10 mmol/L imidazole, 1% DDM, 15% glycerol, and protease inhibitors (pH 8.0). Protein was eluted with PBS buffer adjusted to 300 mmol/L sodium chloride containing 500 mmol/L imidazole, 0.1% DDM, and protease inhibitors (pH 8.0).

**Microarray gene expression analysis**

For the analysis of EDNBR mRNA expression in multiple human tumor and normal biopsy samples (Fig. 1B), the Affymetrix data were obtained from Gene Logic Inc. The analysis shown for probe set ID 204271_s_at was carried out using the HGII133 Plus v2 GeneChip on 3,879 normal human tissue samples (green symbols), 1,605 human cancer tissue samples (red symbols: 1,291 primary and 314 metastatic), and 3,872 human noncancer disease tissue samples (blue symbols). Microarray data were normalized using the Affymetrix MAS (Microarray Analysis Suite) version 5.0 software, with sample expression values scaled to a trimmed mean of 500. The correlation between MITF (probe 226066_at) and EDNBR (204271_s_at) mRNA expression was assessed on the 28 human metastatic melanoma samples contained in these data. For the correlation of MITF and EDNBR in the indicated melanoma cell lines, data were generated at Genentech by using the same Affymetrix probes, chip set, and scaling factors.

**Results**

To identify antibody targets for melanoma, we searched for mRNA transcripts that coded both for cell-surface proteins and exhibited expression levels highly correlated to that of the melanoma oncogene MITF (14). In microarray data generated from a panel of 28 metastatic melanomas, and 18 melanoma cell lines, these criteria were satisfied by mRNA coding for the...
EDNBR (Fig. 1A). An examination of microarray data generated from a large and diverse panel of human tissues revealed that EDNBR mRNA is expressed more highly in melanoma than in the vast majority of all other normal and cancer tissues analyzed (Fig. 1B). In the category labeled bone marrow, a subset of samples exhibiting high levels of EDNBR transcript was further classified as multiple myeloma.

A positive role for EDNBR as a driver of oncogenesis in melanoma is consistent with previous studies of small molecule inhibitors on tumorigenicity of melanoma cell lines in vitro and in vivo (17–19). mAbs offer a complementary therapeutic approach to cell-surface targets and typically operate with greater specificity relative to small molecule inhibitors. Therefore, we attempted to raise therapeutically active antibodies specific to EDNBR. Mice were immunized with intact HEK293 cells overexpressing human EDNBR and the resulting hybridoma supernatants were screened against receptor-positive and -negative cell lines. Antibody 24C7 was of particular interest because its binding to EDNBR was blocked by preincubation of cells with ET-1 (Fig. 2A and B). Moreover, the 24C7 antibody did not cross-react with endothelin A receptor (EDNRA), and by testing various EDNRA/B chimeric receptors, we mapped the binding site to the second extracellular loop of EDNBR (Supplementary Fig. S1). The mutually exclusive binding of 24C7 and ET-1 suggested that the antibody might inhibit receptor-ligand interactions. However, ligand-induced activation of EDNBR signaling, as monitored by MAPK phosphorylation and calcium influx, was unaffected by the 24C7 antibody (Supplementary Fig. S2).

It was possible that the lack of 24C7 binding observed following ET-1 incubation was due to receptor internalization. However, binding of additional distinct antibodies to EDNBR was not affected by preincubation with ET-1 (not shown; Supplementary Fig. S3). Endothelins have been reported to bind their receptors with particularly high affinity (28), which could account for the inability of 24C7 to inhibit receptor activation. Indeed, further analysis of competitive binding revealed that as little as 1.5 nmol/L ET-1 partially blocked binding of 5 nmol/L ET-1 (Fig. 2C). Thus, the binding of 24C7 and ET-1 to EDNBR is mutually exclusive, but the antibody lacks affinity sufficient to compete with the high-affinity binding of ET-1.

Before initiating an effort to obtain more suitable therapeutic antibodies, we sought to more directly assess the role of EDNBR in melanoma growth in vivo. We engineered 2 melanoma cell lines, UACC-257 and A2058, in which EDNBR could be knocked down conditionally by induction of shRNA on the addition of doxycycline (Supplementary Fig. S4). Although administration of doxycycline to animals bearing tumor xenografts derived from these cell lines enhanced progression-free survival and inhibited tumor growth, the effects were rather modest (Supplementary Fig. S4). These data, along with the disappointing results of an endothelin receptor antagonist in phase II melanoma clinical studies (20, 21), dissuaded us from further pursuing an approach to receptor inhibition. However, the exceptional specificity and abundant overexpression of EDNBR in melanoma make it an attractive target for an ADC. Therefore, we generated additional mAbs to EDNBR that would bind with high affinity irrespective of endothelin ligand binding. Immunization of mice with purified recombinant EDNBR protein resulted in numerous EDNBR-specific mAbs, all of which reacted strongly with the N-terminal extracellular sequence.

From this panel, an mAb designated 5E9 was chosen for further study on the basis of its cross-reactivity with rodent, non-human primate, and human EDNBR. The epitope for 5E9 resides between amino acid positions 65 and 101 within the N-terminal extracellular sequence.
(Supplementary Fig. S1), thus precluding any loss of reactivity that might result from proteolysis reported to occur at amino acid R64 (29). Moreover, ET-1 does not compete with binding of 5E9 to EDNBR (Supplementary Fig. S3).

An important property of an ADC is the capacity to deliver the appended drug payload into cells following cell-surface binding. Accordingly, we carried out immunofluorescent staining to examine the uptake of 5E9 into cells expressing EDNBR. The antibody was readily detected in vesicular structures in the fixed permeabilized cells and the staining colocalized with both the lysosomal marker LAMP1 and the early endosomal marker EEA1 (Fig. 3).

The rapid internalization of 5E9 into melanoma cells prompted us to pursue an armed antibody approach. The potent cytotoxic compound MMAE was conjugated to 5E9 by using a linker containing the protease sensitive valine-citrulline dipeptide (26). The ADC was first evaluated in vitro on cell lines expressing increasing levels of EDNBR. To identify and rank EDNBR-positive cell lines, FACS was carried out on a panel of 18 melanoma cell lines (Supplementary Fig. S5). The majority of these cell lines were positive and, on the basis of this survey, we chose 3 cell lines, A2058, WM-266-4, and UACC-257, to represent low, medium, and high EDNBR expression, respectively. By Scatchard analysis, the number of 5E9 binding sites on A2058, WM-266-4, and UACC-257 was estimated at 1,582, 9,410, and 33,939 per cell, respectively, and the dissociation constants ($K_d$) were estimated at 1.4 to 2.4 nmol/L (Supplementary Fig. S6). Titration of these cell lines with the 5E9 ADC showed specific cell killing relative to control ADC that was generally proportional to the level of EDNBR expression (Fig. 4A).

Variables in addition to receptor density, such as response to the released drug, might also contribute to the relative efficacy of the ADC. To address this, we carried out titration of free MMAE with the A2058, WM-266-4, and UACC-257 cell lines and estimated EC$_{50}$ values of 0.28, 0.14, and 0.5 nmol/L respectively, for the 3 cell lines (Fig. 4B). Thus, response to free drug is probably not a significant factor contributing to the potency of the ADC on these cell lines. To further examine the relationship between target density and cell killing, we established a stable derivative of the UACC-257 cell line in which EDNBR levels could be knocked down by conditional expression of shRNA. The total levels EDNBR, and the amount of 5E9 antibody that undergoes internalization, were significantly reduced following induction of the EDNBR-specific shRNA, whereas induction of the control luciferase shRNA had no effect (Fig. 5A). Consistent with the reduction in target levels, cell killing by the ADC was impaired by induction of the EDNBR-specific shRNA but not by induction of the luciferase control shRNA (Fig. 5A).

We also generated a derivative of the A2058 cell line that overexpresses EDNBR. The increase in target expression enhanced cell killing by the ADC by more than 100-fold relative to the parental cell line (Fig. 5B).

Two of the 3 cell lines, A2058 and UACC-257, were found to be competent to generate relatively uniform tumor xenografts in the flanks of athymic mice and were thus chosen for in vivo efficacy studies. To gauge the level of EDNBR expression on these cell lines, relative to that present on human melanomas, a comparative immunohistochemical analysis was carried out. Fixed, paraffin-embedded pellets of the A2058 and UACC-257 cell lines were sectioned and stained with antibody to EDNBR, along with a tissue microarray containing sections from 40 human melanomas. Melanoma sections were assigned scores ranging from 0 to 3 $^{+}$, where 36 of 40 sections were positive with staining intensities of $1^{+}$ or greater (Fig. 6).
Figure 4. Relationship of receptor level to ADC cell killing in vitro. A, the relative amounts of cell-surface EDNBR on melanoma cell lines A2058 (top), WM-266-4 (middle), and UACC-257 (bottom) is shown by flow cytometry on live cells (green line). Secondary antibody alone was used as a control (red line). The indicated number of receptors/cell was estimated separately by Scatchard analysis. Cell killing by 5E9 ADC titration is presented adjacent to each flow cytometry plot for the corresponding cell line. The indicated concentrations of 5E9-vc-MMAE (red line), control IgG-vc-MMAE (blue line), or equivalent amount of PBS vehicle (black line) were incubated with cells for 5 days and relative cell viability (% axis) assessed using CellTiter-Glo. B, the cell lines A2058 (blue), UACC-257 (red), and WM-266-4 (black) were treated with increasing concentrations of free MMAE for 5 days and cell viability was determined by the CellTiter-Glo assay.
renal cancer cell line 786-O, which lacks EDNBR expression, nor was detectable staining observed on EDNBR-positive cell lines or tissues with isotype control antibody (data not shown).

For in vivo efficacy studies, tumor xenografts were grown to an average size of approximately 200 mm³, whereupon animals were randomized into groups of 10 each, with each group receiving a single IV injection of the 5E9 ADC or control ADC. Doses of 1, 3, and 6 mg/kg of 5E9 ADC or 6 mg/kg of control ADC were administered to animals bearing A2058 tumors. A sustained reduction in tumor volume was observed at the high dose of the 5E9 ADC relative to the matching dose of control ADC or vehicle (Fig. 7A). Efficacy was not apparent for the group dosed at 1 mg/kg, and a trend toward growth retardation was noted for the 3 mg/kg dose group. Consistent with in vitro cell killing, the UACC-257 xenograft tumors were more responsive to the

![Image](https://example.com/image1)

Figure 5. Altering EDNBR expression level affects ADC cell killing in vitro. A, Western blot (top left) for EDNBR on total cell lysates from a UACC-257 cell line expressing shRNA specific to EDNBR in response to doxycycline (Dox). These UACC-257 cells and a control cell line expressing shRNA specific to luciferase were treated with or without doxycycline and then fixed and permeabilized prior to immunofluorescent detection of EDNBR (top right). A second set of live cells was subjected to a cell killing assay with 5E9-vc-MMAE (bottom) as described in Figure 4. B, Western blot (top) of total cell lysate from the A2058 parental cell line (P) or a stable derivative overexpressing EDNBR (OE). The two cell lines were subjected to a cell killing assay (bottom) with 5E9-vc-MMAE or control IgG-vc-MMAE as described in Figure 4.

![Image](https://example.com/image2)

Figure 6. Comparative EDNBR expression on human melanoma and melanoma cell lines. Sections prepared from fixed, paraffin-embedded pellets of UACC-257 and A2058 cells and from 40 fixed, paraffin-embedded human melanoma specimens were stained with antibody to EDNBR. A relative staining intensity (0–3+) was assigned and a melanoma specimen representing each intensity level is presented. The number of specimens (n) assigned to each intensity level is shown below.

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For in vivo efficacy studies, tumor xenografts were grown to an average size of approximately 200 mm³, whereupon animals were randomized into groups of 10 each, with each group receiving a single IV injection of the 5E9 ADC or control ADC. Doses of 1, 3, and 6 mg/kg of 5E9 ADC or 6 mg/kg of control ADC were administered to animals bearing A2058 tumors. A sustained reduction in tumor volume was observed at the high dose of the 5E9 ADC relative to the matching dose of control ADC or vehicle (Fig. 7A). Efficacy was not apparent for the group dosed at 1 mg/kg, and a trend toward growth retardation was noted for the 3 mg/kg dose group. Consistent with in vitro cell killing, the UACC-257 xenograft tumors were more responsive to the
ADC and regressed or remained static for the entire study period of more than 100 days in response to single dose of 1 mg/kg 5E9 ADC (Fig. 7B). This result was indistinguishable from that of the 4 mg/kg 5E9 ADC dose group, suggesting that a maximal effect had been achieved at 1 mg/kg. In animals treated with 4 mg/kg of control ADC, the tumors grew at the same rate as those treated with vehicle control. These results suggest that efficacy can be achieved with the 5E9 ADC in tumors that correspond to the full expression range of EDNBR encountered in human melanomas.

Discussion

The EDNBR is overexpressed in human melanoma and attempts to exploit it as a therapeutic target have been reported (6, 30). It is an attractive target because the function of EDNBR in embryonic development and its mode of signaling in cells are consistent with a potential functional contribution to tumor progression (7). Germ-line-inactivating mutations in either EDNBR or ET-3 are responsible for the WS4 variant of Waardenburg syndrome, which is characterized by melanocytic deficits in skin and other tissues (12). The MITF is mutated in another variant of Waardenburg syndrome, WS2, which again is associated with melanocytic deficits (31). Thus, loss of function of either EDNBR or MITF negatively affects the growth and survival of melanocyte progenitors. Conversely, excess activity of these genes could reanimate growth and migratory characteristics of these cells and thereby contribute to tumor progression. Indeed, mutations in MITF, and amplification of the gene coding for it, have been identified in human melanoma (13, 14). Although the gene encoding EDNBR is neither amplified nor sporadically mutated in melanoma, the transcript is overexpressed and we find this correlates with MITF transcript across tumor tissue samples and cancer cell lines. Therefore, we sought to target EDNBR with therapeutic antibodies.

Our first attempt at generating antibodies to EDNBR employed HEK293 cells overexpressing the receptor as the immunogen administered to BALB/c mice. This resulted in mAbs that required the second extracellular loop of the receptor for binding that was blocked on preincubation with endothelin ligands. Nevertheless, these antibodies were incapable of inhibiting EDNBR activation by endothelins. It is possible that the high affinity of ET-1 for EDNBR, with a reported $K_d$ of 80 pmol/L (28), could preclude competition by our antibodies when used at therapeutically relevant concentrations. Therefore, the appropriate antibodies with very high affinity could conceivably interfere with ligand binding to EDNBR. However, before engaging an effort to generate such antibodies, we conducted experiments to further assess the contribution of EDNBR to tumorigenicity.

We carried out conditional knockdown of the EDNBR in 2 independent melanoma cell lines and observed positive effects on survival of tumor-bearing animals and retardation of tumor growth. This was consistent with a previous study in which inhibition of EDNBR impacted the growth of melanoma cell lines in vitro (19). Nevertheless, the marginal impact on tumorigenicity in our studies suggested that more robust interference with EDNBR might be required to significantly effect tumor growth. Indeed, marked inhibition of melanoma xenografts by BQ788, a potent EDNBR inhibitory compound, has been reported (18). Despite these encouraging preclinical results, phase II clinical trials with the endothelin receptor inhibitor bosentan have not produced any objective responses (20, 21). We therefore turned to an approach that exploits the overexpression of EDNBR in melanoma but does not rely on functional interference.

Advances in ADC technology have resulted in encouraging responses in recent clinical trials (32). The development of more potent drugs and linkers with enhanced stability has greatly improved the prospects for this approach. This has coincided with advances in high-
throughput technologies, enabling the identification of highly specific cell-surface antigens expressed on tumor cells. Our gene expression analysis, in which thousands of human tumor and normal tissue samples are represented, revealed overexpression of EDNBR mRNA in melanoma that greatly exceeded expression in any other normal tissue.

Our selection of the 5E9 mAb for drug conjugation was based on high-affinity binding, cross-species reactivity, lack of interference by endothelins, and a high rate of cellular internalization. When conjugated with MMAE through the valine-citrulline peptide linker, the 5E9 ADC exhibited remarkable potency. Notably, the growth of the A2058 melanoma tumor xenograft was strongly retarded by a single dose of 6 mg/kg 5E9-vc-MMAE despite the estimated expression of only 1,500 copies of EDNBR per cell. The UACC-257, which expresses 30,000 copies per cell, exhibited no signs of growth to 100 days following a single administration of 1 mg/kg 5E9-vc-MMAE. On the basis of EDNBR expression, the A2058 and UACC-257 represent the low and high expressers, respectively, relative to our panel of 40 human melanomas.

Although the density of EDNBR expression on melanoma cells is a primary factor that will influence efficacy, it is likely that additional variables will also have an impact. For example, the efficiency by which cancer cells generate the active drug from the internalized ADC may vary across individual cancers. The drug substance that is ultimately released from the ADC will also be subject to metabolism and efflux that will vary with the genetic background of the individual cancer and, possibly, that of the patient. Moreover, acquired resistance to the drug might arise to varying degrees by selection during the course of treatment. Selection against the antibody target represents an additional mode of acquired resistance. However, we have monitored EDNBR expression in our preclinical efficacy studies and have not observed downregulation of the receptor following prolonged ADC exposure.

Melanoma remains one of the most aggressive human cancers for which effective treatment options are extremely limited. Overall, our efficacy studies indicate that EDNBR is an excellent target for the application of ADCs in melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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An Antibody–Drug Conjugate Targeting the Endothelin B Receptor for the Treatment of Melanoma

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