An antibody-drug conjugate targeting the endothelin B receptor for the treatment of melanoma.

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Translational Significance

Targeted cancer therapies exploit features unique to the cancer cell and thereby minimize damage to normal tissues. Accordingly, we have targeted the Endothelin B receptor based on its overexpression on human melanoma cells. This receptor is particularly attractive as a target for antibody-drug conjugate therapy due to its minimal expression on normal tissue, its cell surface localization and rapid endocytosis. The receptor was expressed on the vast majority of melanoma tissue specimens examined and the antibody-drug conjugate demonstrates preclinical efficacy even in a model representing the low end of the expression range. As a platform, antibody-drug conjugates, such as SGN-35 and TDM1, have recently demonstrated very encouraging results in clinical trials. Our preclinical results suggest that the antibody-drug conjugate targeting the endothelin B receptor may provide an effective new therapy for melanoma patients.
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 (EDNRB), was first analyzed for a functional contribution to tumor growth using conditional induction of shRNA. Secondly, antibodies were raised to the receptor, conjugated with monomethylauristain A 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 (mAb) reactive with the N-terminal tail of EDNRB was found to internalize rapidly into melanoma cells. When conjugated with monomethylauristatin E (MMAE), the antibody-drug conjugate (ADC) demonstrated 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 EDNRB 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 endothelin B receptor is highly efficacious in preclinical models of melanoma.
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, as well as adoptive immunotherapy, are currently in clinical testing for metastatic melanoma (2). The results from some of these recent studies appear encouraging, but a durable impact on overall survival will likely require therapeutic combinations including additional new agents.

Over 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 (EDNRB) is critical for the faithful derivation of melanocytic cells emanating from the neural crest during embryonic development (6, 7). Melanocyte precursors rely upon EDNRB activity to proliferate and migrate from the neural tube to their final destinations (8, 9). Mice with defective genes coding
for either EDNRB 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 EDNRB (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 EDNRB activity to the fate of melanoblasts underscores a potential role for this receptor in the progression of melanoma. The expression of EDNRB mRNA and protein was reported to increase during disease progression from dysplastic nevi to metastatic melanoma (16). Blockade of EDNRB activity by two independent small molecule inhibitors interfered with growth and survival of melanoma cells and tumor xenografts (17-19). These preclinical studies implicate EDNRB as a potential driver of melanoma progression. However, translation of EDNRB 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 performed a search for transcripts encoding cell surface proteins with expression correlative to that of MITF, the melanoma oncogene. Accordingly, EDNRB was readily
identified in a panel of metastatic melanomas and melanoma cell lines. Herein we describe the application of antibodies to EDNRB and, in particular, report that EDNRB is an extremely efficient target for antibody-drug conjugate therapy.

**Materials and Methods:**

**EDNRA and EDNRB cDNAs**

Human EDNRA cDNA from Origene (Rockville, MD), human EDNRB cDNA from Incyte (Wilmington, DE), mouse EDNRB cDNA from I.M.A.G.E.(Invitrogen, Carlsbad, CA), cynomologus monkey EDNRB cDNA from liver and lung cDNA libraries and rat EDNRB cDNA from neonatal cDNA library were cloned into a CMV promoter driven N-terminal FLAG-tagged vector. These constructs were used to generate Δ64EDNRB lacking the N-terminal 64 amino acids and the EDNRA/EDNRB chimeras in which one of each of the 4 extracellular domains of EDNRA was substituted into EDNRB. The N-His-ΔSP-EDNRB cDNA coding for N-terminal 8XHis-tagged EDNRB protein without native signal peptide was sub-cloned into Baculovirus vector pAcGP67-B (BD BioSciences, San Jose, CA) and employed for production of immunogen used to raise the 5E9 antibody.

**Immunological procedures**

For production of monoclonal antibodies, BALB/c mice were immunized with either HEK293 cells stably transfected with full-length EDNRB clone or with purified EDNRB protein. Immunization, hybridoma selection and antibody
purification were performed 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% Fetal Bovine Serum (FBS). All subsequent steps were carried out at 4°C. Cells were incubated for 1 h each with 2-5 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-EDNRB antibodies (5E9 and 24C7) for EDNRB cell surface detection, FLAG M2 mAb (F-3165, Sigma Life Science, St. Louis, MO) for N-Term FLAG tag detection were used. Alexa488 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-EDNRB in-house generated mAb 1H1.8.5, rabbit polyclonal anti-GAPDH antibody (PA1-987, Affinity Bioreagents, Rockford, IL), p44/42 MAP kinase and Phospho p44/42 MAP kinase (Thr202/Tyr 204) (9102, 9101S, Cell Signaling Technology, Danvers, MA), and β-Tubulin (556321, BDPharmingen/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 Immunoclipon Chemicals for Research, Gilbertsville, PA) were used.
For immunofluorescence, 10,000 cells were plated on four-well chamber slides and incubated for 72 h at 37°C at 5% CO₂. Cells were fixed with 4% Ultra pure Formaldehyde (18814, Polysciences Inc, Warrington, PA) and permeabilized with 0.05% saponin (S-4521, Sigma Life Science). Non-specific signal was blocked using PBS containing 5% goat serum (G-9023, Sigma Life Science) and primary and secondary antibodies were applied to cells in PBS containing 2% goat serum. Live cells were incubated with 5 μg/mL anti-EDNRB antibodies for 1 h on ice for surface expression detection. Internalization of antibodies was evaluated by applying 2 μg/mL anti-EDNRB chimeric antibody 5E9 for 2 h at 37°C to live cells in complete medium containing 25 μg/mL Leupeptin and 10 μg/mL Pepstatin (Roche Applied Science, Indianapolis, IN). Localization of internalized EDNRB was further determined on these cells by incubating fixed and permeabilized cells for 1 h at room temperature with antibodies for organelle markers; 4 μg/mL antibody for Lysosomal Associated Membrane Protein 1, (L1418, Sigma Life Science) or 5 μg/mL Early Endosomal Antigen 1, EEA1, (324610, EMD BioScieces, Gibbstown, NJ). Cy³ conjugated anti-mouse or anti-human detection reagents (115-165-166, 109-165-098, Jackson ImmunoResearch, West Grove, PA) and Alexa 488 conjugated anti-rabbit detection reagent (A11008, Invitrogen) were used. Cells were mounted with VectaShield (H-1200, Vector Labs, Inc, Burlingame, CA) and sealed with nail polish. Images are taken at 60X magnification using Nikon microscope.

EDNRB immunohistochemistry on tissue sections was performed on the Ventana Discovery XT Platform using CC1 standard antigen retrieval as a pretreatment.
Sections were incubated with goat-anti-human-EDNRB primary antibody (sc-21196, Santa Cruz Biotechnologies, Santa Cruz, CA) at 0.05 μg/mL (1:4000) for 60 minutes at 37°C followed by incubation with an unconjugated rabbit-anti-goat-IgG secondary antibody (Vector Labs, Burlingame, CA) at 5 μg/mL (1:200) for 32 minutes at room temperature. Sections were subsequently incubated for 16 minutes with anti-rabbit-OMNIMAP-HRP reagent (Ventana Medical Systems, Tuscon, AZ) followed by Ventana DAB colorometric 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 isotypic control. The human melanoma tissue samples were purchased from US Biomax, Inc. (Rockville, MD). The overall staining intensity of tissue samples was scored as none (0), weak (1+), moderate (2+) or strong (3+).

The determination of anti-EDNRB binding sites per cell was performed 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 (Manassas, VA) or NCI-60, (Frederick, MD) and the cell lines 526mel, 537 mel, 624mel, 888mel, 928 mel and 1300mel 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.
mediated knockdown was induced in clonal populations by culturing these cells for 72 h in complete medium containing 2 μg/mL doxycycline (631311, Clontech Laboratories, Mountain View, CA). shRNA-bearing clonal populations were selected by the addition of 1-5 μg/mL puromycin (631306, Clontech Laboratories) as appropriate. Stable HEK293 transfected populations were selected using 500 μg/mL Geneticin (10131-027, Gibco/Invitrogen). Cells were transfected with DNA using Lipofectamine 2000 (11668019, Invitrogen) as per the recommended protocol. To assess the effects of antibody-drug conjugates 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 were added to triplicate wells. Five days later, cell survival was determined using CellTiter-Glo Luminescent Cell Viability Reagent (G7572, Promega Corporation, Madison, WI) and with an EnVision 2101 Mutilabel Reader (Perkin-Elmer, Waltham, MA).

Cell lines with inducible shRNA-bearing lentivirus constructs were generated essentially as described previously (23-25). shRNA targeting EDNRB at nucleotides 1363-1381 of NM_000115, target sequence 5’-TCACTGAATTCTGCATTA-3’, (D-003657-02, Dharmacon RNAi Technologies, Lafayette, CO) was used to knockdown EDNRB in melanoma cell lines. The experiments were controlled using shRNA targeting Luciferase gene. Mutant EDNRB gene, impervious to knockdown by the above shRNA, was generated by changing target nucleotide sequence to 5’-AGCTTTAAACAGTTGTATCA-3’ while maintaining the protein identity.
MAPK phosphorylation and calcium influx assays

For MAPK phosphorylation, 2 X 10^6 UACC-257 cells were serum starved for 24 h on a 60 mm dish. Antibody 24C7 (5μg/mL) or BQ788 Sodium salt (100 nM; PED-3788-PI, Peptide International Inc, Louisville, KY) was added and the cells incubated for 1 h at 37°C prior to addition of 100 nM human ET-1 (PED-4198-v, Peptide International) for 5 minutes at 37°C. Cell lysates were probed for total or phosphorylated p44/42 MAP kinase and for β-Tubulin. Protein bands were quantitated using Odyssey infrared imaging system from Li-Cor Biosciences (Lincoln, NE). Phosphorylated p44/42 (Thr202/Tyr204) MAP kinase bands were normalized to total p44/42 MAP kinase and the β-Tubulin loading control.

For calcium influx, 25,000 HEK-293 cells transfected with EDNRB were plated on a 96-well black sided plate and incubated at 37°C, 5% CO₂. After 48 h, medium was aspirated and cells were loaded with dye for 2 h at 37°C using FLIPR Calcium 3 assay kit (# R8090, Molecular Devices, Sunnyvale, CA). Loading dye was spiked with 5 μg/mL 24C7 Ab or 100 nM 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 upon addition of 100nM 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, "Guide for the Care and Use of Laboratory Animals", (NIH Publication #85-23). Washington (DC): National Academy Press; 1996.

Antibodies were conjugated with monomethylauristatin E (MMAE) as described previously (26). The drug to antibody ratio for 5E9vcMMAE ranged from 2.95 to 3.55 and the drug to antibody ratio for control IgGvcMMAE ranged from 2.5 to 3.2

Average tumor volumes with standard deviations were determined from 10 animals per group. For efficacy studies with ADCs, 6-8 week old female CRL Nu/Nu mice from Charles River Laboratories were inoculated subcutaneously in the dorsal right flank with 10 million UACC-257 or with 5 million A2058 cells in HBSS with Matrigel. When tumor volumes reached ~200 mm$^3$ (day 0), animals were randomized into groups of 10 each and administered a single IV injection of anti-EDNRB chimeric or monoclonal antibody conjugated to MMAE through the valine citrulline linker (26). MMAE conjugated to anti-gD antibody (human isotype) or to anti-ragweed antibody (mouse isotype) were used as controls. Tumor volumes were measured twice per week until study end. The same procedure was followed for growth studies involving A2058 and UACC-257 cell lines expressing inducible shRNA, except that when tumor volumes reached ~200 mm$^3$ (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 EDNRB 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), 15% glycerol and nutating the mixture overnight at 4°C. Supernatent was collected after high-speed centrifugation and adjusted to 300 mM sodium chloride concentration and pH 8.0. The sample was loaded on to Ni-NTA Superflow column (30410, Qiagen, Valencia, CA). The column was washed with PBS buffer adjusted to 300 mM Sodium chloride, 10 mM imidazole, 1% DDM, 15% glycerol, protease inhibitors, (pH 8.0). Protein was eluted with PBS buffer adjusted to 300 mM sodium chloride containing 500 mM imidazole, 0.1% DDM and protease inhibitors (pH 8.0).

Microarray gene expression analysis

For the analysis of EDNRB mRNA expression in multiple human tumor and normal biopsy samples (Fig. 1B), the Affymetrix data were obtained from Gene Logic, Inc. (Gaithersburg, MD). The analysis shown is for probe set ID 204271_s_at, performed using the HGU133 Plus v2 GeneChip on 3879 normal human tissue samples (green symbols), 1605 human cancer tissue samples (red symbols: 1291 primary and 314 metastatic), and 3872 human non-cancer 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 EDNRB (204271_s_at) mRNA expression was performed on the 28 human metastatic melanoma samples contained in this data. For the correlation of MITF and EDNRB in the indicated melanoma cell lines, data was generated at Genentech 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 endothelin B receptor (Fig. 1A). An examination of microarray data generated from a large and diverse panel of human tissues revealed that EDNRB 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 EDNRB transcript were further classified as Multiple Myeloma.
A positive role for EDNRB 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). Monoclonal antibodies 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 EDNRB. Mice were immunized with intact HEK293 cells overexpressing human EDNRB and the resulting hybridoma supernatants were screened against receptor-positive and negative cell lines. Antibody 24C7 was of particular interest since its binding to EDNRB 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, and by testing various EDNRA/B chimeric receptors, we mapped the binding site to the second extracellular loop of EDNRB (Fig. S1). The mutually exclusive binding of 24C7 and ET-1 suggested the antibody might inhibit receptor-ligand interactions. However, ligand-induced activation of EDNRB signaling, as monitored by MAP kinase phosphorylation and calcium influx was unaffected by the 24C7 antibody (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 EDNRB were not affected by preincubation with ET-1 (not shown and 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 nM ET-1 partially blocked binding of 5 μg/mL (~30 nM) 24C7 whereas 6 nM ET-1 completely blocked it (Fig. 2C). Thus the binding of 24C7 and ET-1 to EDNRB is mutually exclusive, but the antibody lacks affinity sufficient to compete with the high affinity binding of ET-1.

Prior to initiating an effort to obtain more suitable therapeutic antibodies, we sought to more directly assess the role of EDNRB in melanoma growth in vivo. We engineered two melanoma cell lines, UACC-257 and A2058, in which EDNRB could be knocked down conditionally by induction of shRNA upon addition of doxycycline (Fig. S4). Although administration of doxycycline to animals bearing tumor xenografts derived from these cell lines cells enhanced progression free survival and inhibited tumor growth, the effects were rather modest (Fig. S4). This 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 EDNRB in melanoma makes it an attractive target for an antibody-drug conjugate (ADC). Therefore, we generated additional mAbs to EDNRB that would bind with high affinity irrespective of endothelin ligand binding. Immunization of mice with purified recombinant EDNRB protein resulted in numerous EDNRB-specific mAbs, all of which reacted strongly with the N-terminal extracellular sequence.
From this panel, a mAb designated 5E9 was chosen for further study based on its cross-reactivity with rodent, non human primate and human EDNRB. The epitope for 5E9 resides between amino acid positions 65 and 101 within the N-terminal extracellular sequence (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 EDNRB (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 performed immunofluorescent staining to examine the uptake of 5E9 into cells expressing EDNRB. The antibody was readily detected in vesicular structures in the fixed permeabilized cells and the staining co-localized with both the lysosomal marker LAMP-1 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 monomethyl auristatin E (MMAE) was conjugated to 5E9 using a linker containing the protease sensitive valine-citrulline dipeptide (26). The antibody-drug conjugate (ADC) was first evaluated in vitro on cell lines expressing increasing levels of EDNRB. To identify and rank EDNRB positive cell lines, fluorescent activated cell sorting was performed on a panel of 18 melanoma cell lines (Fig. S5). The majority of these cell lines were positive and, based on this survey, we chose three cell lines, A2058, WM-266-4 and UACC-257, to represent low, medium and high EDNRB expression, respectively. By Scatchard analysis, the number of 5E9 binding sites on A2058, WM-266-4 and UACC-257, was estimated at 1582, 9410, and 33,939.
per cell, respectively, and the dissociation constants were estimated at 1.4 nM to 2.4 nM (Fig. S6). Titration of these cell lines with the 5E9 ADC demonstrated specific cell killing relative to control ADC that was generally proportional to the level of EDNRB 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 performed a titration with free MMAE on the A2058, WM-266-4 and UACC-257 cell lines and estimated EC50s of 0.28, 0.14 and 0.5 nM respectively, for the three 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 ENDRB levels could be knocked down by conditional expression of shRNA. The total levels ENDRB, as well as the amount of 5E9 antibody that undergoes internalization, was significantly reduced following induction of the ENDRB-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 ENDRB-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 ENDRB. The increase in target expression enhanced cell killing by the ADC by over 100-fold relative to the parental cell line (Fig. 5B).

Two of the three cell lines, A2058 and UACC-257, were found 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 EDNRB 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 EDNRB 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). The staining of the two cell lines is shown above the stained melanoma sections representative of each of the four staining intensity levels. The A2058 and UACC-257 expressed levels of EDNRB comparable to 1+ and 3+ melanomas, respectively. No anti-EDNRB staining was detected with the renal cancer cell line 786-O, which lacks EDNRB expression, nor was detectable staining observed on EDNRB 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 mm$^3$, whereupon animals were randomized into groups of 10 each, with each group receiving a single intravenous 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 over 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 EDNRB encountered in human melanomas.

Discussion

The endothelin B receptor 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 EDNRB in embryonic development and its mode of signaling in cells are consistent with a potential functional contribution to tumor progression (7). Germline inactivating mutations in either EDNRB 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 Microphthalmia-Associated Transcription factor MITF is mutated in another variant of Waardenburg syndrome, WS2, which again is associated with melanocytic deficits (31). Thus, loss of function of either EDNRB 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 EDNRB 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 EDNRB with therapeutic antibodies.

Our first attempt at generating antibodies to EDNRB 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 upon preincubation with endothelin ligands. Nevertheless, these antibodies were incapable of inhibiting EDNRB activation by endothelins. It is possible that the high affinity of ET-1 for EDNRB, with a reported Kd of 80 pM (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 EDNRB. However, prior to engaging an effort to generate such antibodies we performed experiments to further assess the contribution of EDNRB to tumorigenicity.

We performed conditional knockdown of the EDNRB in two 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 EDNRB 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 EDNRB might be required to significantly effect tumor growth. Indeed, marked inhibition of melanoma xenografts by BQ788, a potent EDNRB inhibitory compound, have 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 EDNRB in melanoma, but does not rely upon functional interference.

Advances in antibody drug conjugate technology have resulted in encouraging responses in recent clinical trials (32). The development of more potent drugs as well as 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 EDNRB 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 of 5E9vcMMAE, despite the estimated expression of only 1500 copies of EDNRB per cell. The UACC-257, which expresses 30,000 copies per cell, exhibited no signs of growth out to 100 days following a single administration of 1 mg/kg 5E9vcMMAE. On the basis of EDNRB 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 EDNRB 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 EDNRB expression in our preclinical efficacy studies and have not observed down regulation 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 EDNRB is an excellent target for the application of antibody-drug conjugates in melanoma.
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References


Figure Legends:

Figure 1: Expression of EDNRB mRNA. Measurements were performed on the Affymetrix U133P chip and are expressed as scaled average difference. A. Relative expression of EDNRB mRNA (Y-axis) and MITF (x-axis) for 18 melanoma cells lines (top) and 28 human metastatic melanomas. B. Relative expression of EDNRB in human tissues. Each dot represents a normal (green), tumor (red) or diseased non-tumor (blue) human tissue specimen. Rectangles encompass the 25-75-percentile range for each distribution.

Figure 2: Endothelin-1 competes with antibody 24C7. A and B. UACC-257 cells were incubated with or without 100 nM ET-1 for 45 minutes followed by 5 μg/mL of 24C7. Antibody binding was detected by flow cytometry (A) or immunofluorescence (B). C. HEK293 cells ectopically expressing EDNRB were incubated for 45 minutes with the indicated concentrations of ET-1 followed by 5 μg/mL 24C7. Flow cytometry data represents binding by antibody (black line) or secondary antibody only (gray line).

Figure 3. Cell internalization of EDNRB antibody 5E9. Live UACC-257 cells were incubated with 2 μg/mL of 5E9 antibody for 2 h at 37°C and then fixed and co-stained with antibody to either Lysosomal Associated Membrane Protein 1 (LAMP1; top, middle panel) or Early Endosomal Antigen 1 (EEA1; bottom middle panel). 5E9 was detected with Cy3-labelled secondary antibody (red) and antibodies to LAMP1 and EEA1 with Alexa 488-labelled secondary antibody (green). Merged images are presented in panels at right.

Figure 4. Relationship of receptor level to ADC cell killing in vitro. A. The relative amounts of cell surface EDNRB on melanoma cell lines A2058 (top), WM-266-4 (middle) and UACC-257 (bottom) is demonstrated 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 (y-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.

Figure 5. Altering EDNRB expression level affects ADC cell killing in vitro. A. Western blot (top left) for EDNRB on total cell lysates from a UACC-257 cell
line expressing shRNA specific to EDNRB 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, then fixed and permeabilized prior to immunofluorescent detection of ENDRB (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 ENDRB (OE). The two cells lines were subjected to a cell killing assay (bottom) with 5E9-vc-MMAE or control IgG-vc-MMAE as described in figure 4.

**Figure 6. Comparative EDNRB 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 EDNRB. 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.

**Figure 7. In vivo efficacy of anti-EDNRB ADC.** Subcutaneous tumors were established in mice inoculated with A2058 (A) or UACC-257 (B) cells. When tumor volumes reached ~200 mm³ (day 0), animals were given a single intravenous injection of either control ADC (IgG-vcE) or anti-EDNRB ADC (5E9-vcE) at the indicated doses. Average tumor volumes with standard deviations were determined from 10 animals per group.
Supplemental figure legends

**Figure S1. Mapping of antibody sites for 5E9 and 24C7.** Chimeric cDNAs were generated wherein each of the 4 extracellular domains of the endothelin A receptor were substituted for the corresponding ECD in the endothelin B receptor (ECD1-4A). Additional constructs include the full length endothelin A and B receptors (FL-EDNRB, FL-EDNRA) and EDNRB lacking 64 amino terminal residues (Δ64 EDNRB) The N-terminal FLAG epitope was incorporated into each construct for use as a positive control. Flow cytometry for antibodies 5E9, 24C7 and anti-FLAG (black lines) or secondary antibody only (gray lines) is presented.

**Fig S2- Effect of antibody 24C7 on the induction MAPK phosphorylation and calcium flux.** A. UACC-257 cells stably expressing EDNRB were pretreated with either antibody 24C7, endothelin receptor antagonist BQ788 or no addition prior to stimulation by 100 nM ET-1 for 5 minutes. The ratio of phosphorylated MAPK to total MAPK (perk/erk) normalized to tubulin was determined by imaging immunoblots of total cell lysates. B. HEK-293 cells stably overexpressing EDNRB were loaded with dye for 2 hours in the presence of 5 μg/ml 24C7, 100 nM BQ788 or no addition and then stimulated with 100 mM ET-1. Calcium mobilization was determined on a FLIPR High throughput Cellular Screening system.

**Figure S3. Endothelin-1 does not compete with antibody 5E9.** UACC-257 cells were incubated on ice with or without 100 nM ET-1 for 45 minutes followed by 5 μg/mL of 5E9 for 45 minutes. Antibody binding was detected by flow cytometry (A) or immunofluorescence (B). Flow cytometry data represents binding by antibody (black line) or secondary antibody only (gray line).

**Figure S4. Effect of EDNRB knockdown on tumorigenicity.** A. Kaplan Meier Analysis of progression-free survival of mice bearing tumor xenografts from UACC-257 cell populations transduced with lentivirus bearing shRNA targeting either Luciferase (blue and red lines) or EDNRB (orange and green lines). Mice were treated either with 5% sucrose (blue and green lines) or 1 mg/mL Doxycycline in 5% sucrose (red and orange lines). B. Mean tumor volume of xenografts from A2058 cell populations transduced with lentivirus bearing shRNA targeting either Luciferase (Li) or EDNRB (Ei); or populations doubly transduced with lentivirus bearing shRNA targeting EDNRB and mutant shRNA-resistant EDNRB (Ei+R) bearing lentivirus. Mice were treated either with 5% Sucrose water (S) or 1 mg/mL Doxycycline in 5% sucrose water (D). Immunoblots in A and B demonstrate the effect of doxycycline-induced shRNA, and the rescue cDNA, on the levels of EDNRB protein (red) and loading control GAPDH protein (green) for the corresponding melanoma cell types.

**Figure S5. Expression of EDNRB on melanoma cell lines.** The indicated melanoma cell lines were subjected to fluorescence activated flow cytometry using antibody 5E9 (green line) or secondary antibody only (red line).
Figure S6. Estimation of binding site number and affinity constant for antibody 5E9 on melanoma cell lines. A fixed concentration of $^{125}$I-labelled 5E9 antibody was combined with increasing concentrations of unlabelled 5E9 and incubated on ice with the indicated cell lines. Scatchard plots generated from duplicate analysis of UACC-257 (top), WM-266-4 (middle) and A2058 (bottom) are shown.
Fig 1

A

EDNRB

Cell lines

Tumors

Signal Intensity (Ave. Diff. x 10^{-3})

0 5 10 15 20 25 30 35

Adipose
Adrenal
Blood vessel
Bone
Bone marrow
Brain
Breast
Cervix
Colorectal
Endometrium
Esophagus
Gallbladder
Head&Neck
Heart
Kidney
Liver
Lung
Lymphoid
Muscle
Myometrium
Nerve
Neurocrine
Ovary
Pancreas
Placenta
Prostate
Skin
Sm. intestine
Soft tissue
Stomach
Testis
Thymus
Thyroid
Urinary
WBC

MITF

Research.
Fig 2

**A**

![Graph A](image)

**B**

![Image B](image)

**C**

![Graph C](image)
Fig 3

EDNRB  LAMP1/EEA1  Merge

20μM
Fig 4

A

Receptors/cell 1,582

A2058

Receptors/cell 9,410

WM-266-4

Receptors/cell 33,939

UACC257

B

Percent Survival

Free MMAE (nM)

UACC-257

WM-266-4

A2058
A Fig 5

EDNRB Luciferase

shRNA: EDNRB
Dox: - +

EDNRB
GAPDH-

+ DOX
- DOX

B

EDNRB
GAPDH-

P OE

Ab concentration (μg/mL)

Percent Survival

0 001 0.01 0.1 1 10

Luc - Dox
Luc +Dox
EDNRB -Dox
EDNRB +Dox

Ab concentration (μg/mL)

Percent Survival

0 001 0.01 0.1 1 10

PBS (OE)
IgG-vc-MMAE (OE)
5E9-vc-MMAE (P)
5E9-vc-MMAE (OE)
Fig 6

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Fig. S1

![Diagram showing molecular structures with labels ECD1, ECD2, ECD3, ECD4, and FLAG-N.](image)

- **5E9**
  - ECD1-A
  - ECD2-A
  - ECD3-A
  - ECD4-A
  - FL EDNRA
  - Δ64 EDNRB

- **24C7**
  - ECD1-A
  - ECD2-A
  - ECD3-A
  - ECD4-A
  - FL EDNRA
  - Δ64 EDNRB

- **anti-FLAG**
  - ECD1-A
  - ECD2-A
  - ECD3-A
  - ECD4-A
  - FL EDNRA
  - Δ64 EDNRB
Fig. S2

A

Perk/erk
Normalized to tubulin

No mAb  24C7.4  BQT88

-ET1  +ET1

B

Endothelin-1
Endothelin-1 + 5μg/ml 24C7.4
Endothelin-1 + 100nM BQT88
Fig. S3

A

\begin{align*}
\text{100nM ET-1} \\
\end{align*}

\begin{align*}
\text{NA} \\
\end{align*}

B

\begin{align*}
\text{- ET-1} \\
\text{+ ET-1} \\
\text{20µM} \\
\end{align*}
Fig. S4

A

shRNAi: EDNRB Luciferase
Dox: + + - +

50kD-
GAPDH-

Progression free survival (%)

DAY

1 11 21 31 41 51 61

B

shRNAi: EDNRB Luciferase cDNA+
Dox: + + - + - +

50kD-
GAPDH-

Tumor Volume (mm³)

DAY

0 5 10 15

0 500 1000 1500 2000 2500

Ei+S
Ei+R+S
Li+S
Li+D
Ei+R+D
Ei+D
Fig. S5

526mel  537mel  888mel  928mel

A2058  COLO 829  G-361  Hs-294-T

SK-MEL-5  SK-MEL-5(PRC)  SK-MEL-28  WM-266-4

1300mel  A375  Malme-3M  SK23

UACC-257  UACC-257(NCI60)

Alexa 488 conjugated 2^{0} Ab

2 \mu g/mL 5E9
**Fig. S6**

Assay 1: 5E9.UACC257  
KD = 1.9nM with 32,634 sites/cell

Assay 2: 5E9.UACC257  
KD = 2.1nM with 35,244 sites/cell

Assay 1: 5E9.WM26-64  
KD = 1.4nM with 8686 sites/cell

Assay 2: 5E9.WM26-64  
KD = 1.6nM with 10,133 sites/cell

Assay 1: 5E9.A2058  
KD = 2.2nM with 1534 sites/cell

Assay 2: 5E9.A2058  
KD = 2.4nM with 1630 sites/cell
An antibody-drug conjugate targeting the endothelin B receptor for the treatment of melanoma.

Jyoti Asundi, Chae Reed, Jennifer Arca, et al.

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