CR011, a Fully Human Monoclonal Antibody-Auristatin E Conjugate, for the Treatment of Melanoma

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Abstract **Purpose:** Advanced melanoma is a highly drug-refractory neoplasm representing a significant unmet medical need. We sought to identify melanoma-associated cell surface molecules and to develop as well as preclinically test immunotherapeutic reagents designed to exploit such targets.
Experimental Design and Results: By transcript profiling, we identified *glycoprotein NMB* (*GPNMB*) as a gene that is expressed by most metastatic melanoma samples examined. GPNMB is predicted to be a transmembrane protein, thus making it a potential immunotherapeutic target in the treatment of this disease. A fully human monoclonal antibody, designated CR011, was generated to the extracellular domain of GPNMB and characterized for growth-inhibitory activity against melanoma. The CR011 monoclonal antibody showed surface staining of most melanoma cell lines by flow cytometry and reacted with a majority of metastatic melanoma specimens by immunohistochemistry. CR011 alone did not inhibit the growth of melanoma cells. However, when linked to the cytotoxic agent monomethylauristatin E (MMAE) to generate the CR011-vcMMAE antibody-drug conjugate, this reagent now potently and specifically inhibited the growth of GPNMB-positive melanoma cells *in vitro*. Ectopic overexpression and small interfering RNA transfection studies showed that GPNMB expression is both necessary and sufficient for sensitivity to low concentrations of CR011-vcMMAE. In a melanoma xenograft model, CR011-vcMMAE induced significant dose-proportional antitumor effects, including complete regressions, at doses as low as 1.25 mg/kg.
Conclusion: These preclinical results support the continued evaluation of CR011-vcMMAE for the treatment of melanoma.

Melanoma is a common neoplasm and its incidence is increasing worldwide at a dramatic rate (1). Melanoma accounts for only 4% of skin cancer cases yet causes ~79% of all skin cancer deaths. In 2004, an estimated 55,100 Americans were diagnosed with melanoma and ~7,910 would die of the disease (2). An increasing frequency of newly diagnosed melanomas, ranging from 3% to 8% annually, has also been observed worldwide (3, 4).

Therapeutic options for patients with late-stage melanoma presenting with regional and/or distant metastases are limited. Dacarbazine is the only cytotoxic drug currently approved by the Food and Drug Administration for the treatment of stage IV metastatic melanoma, with a response rate of <15% and a median response duration of 4 to 5 months (5). The majority of polychemotherapy regimens failed to show significant survival benefits (6), nor did the use of adjuvant therapeutic agents such as IFN-α and interleukin 2, which pose severe toxicity (7, 8). The poor efficacy and adverse side effects of available therapies has led to a considerable interest in the development of alternative therapies, such as monoclonal antibodies (mAb), for the treatment of metastatic melanoma (9).

Recent advances in genetic engineering have significantly decreased antibody immunogenicity and increased antibody half-life (10, 11). Antibody-based therapeutics, such as Rituxan, Herceptin, and Avastin, have recently enjoyed clinical success in the treatment of some hematopoietic malignancies and solid tumors. Although antibodies that target tumor or its vasculature may be useful in an unconjugated form, it is sometimes advantageous to couple a tumor-targeting antibody to an isotope (e.g., Zevalin and Bexxar) or to a cytotoxic compound (e.g., Mylotarg). This strategy allows for the selective delivery of cytotoxic agents to the tumor with the goal of reducing the toxicity that is often associated with the systemic administration of cytotoxic agents while preserving or enhancing the antitumor activity of these agents.

Our genome-wide transcript expression profiling, coupled with a systems biology analysis of human melanoma clinical

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Received 9/16/05; revised 12/9/05; accepted 12/14/05.
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doi:10.1158/1078-0432.CCR-05-2018

specimens and cell lines, led to the identification of a tumor-associated protein, called glycoprotein NMB (GPNMB), as a potential target that can be exploited for the treatment of melanoma. GPNMB is predicted to be a 560-amino-acid type I transmembrane protein with closest homology (26% amino acid identity) to the melanocyte/melanoma–specific protein, pMEL17 (12). The normal function of human GPNMB is unknown, and orthologues have been isolated from mouse (DC-HIL; ref. 13), rat (Osteoactivin; ref. 14), and quail (QNR-71; ref. 15).

Previous investigations have associated GPNMB expression and function with cancer. *GPNMB* was first identified as a gene that was differentially expressed among melanoma cell lines with high and low metastatic potential (12) and was subsequently identified as a candidate glioma tumor marker due to its high transcript expression in this tumor type and restricted normal tissue distribution (16). GPNMB expression has also been described in liver cancer, squamous cell lung carcinoma, and soft tissue tumors (17–19). Moreover, ectopic expression of GPNMB in cancer cells increased their *in vitro* invasiveness and promoted their metastasis *in vivo* (17, 20). Finally, GPNMB was shown to interact with the surface of endothelial cells (13), a finding that may have implications for GPNMB-expressing melanoma cell transendothelial migration and metastasis.

To explore the potential utility of GPNMB as a target for melanoma therapy, fully-human mAbs were generated to this protein. The lead mAb, CR011, was characterized and coupled to the dolastatin-10-related cytotoxic drug monomethylauristatin E (MMAE), a potent inhibitor of mitotic spindle formation (21). The resulting antibody-drug conjugate, designated CR011-vcMMAE, was evaluated for growth-inhibitory activity on melanoma cell lines *in vitro* and for activity against melanoma xenografts *in vivo*. The results presented in this study suggest that GPNMB represents a promising target for the identification and treatment of advanced melanoma and that CR011-vcMMAE is worthy of continued therapeutic evaluation.

Materials and Methods

Cell lines and transfections. M14, UACC-257, and LOXIMVI cell lines were obtained from the National Cancer Institute (Bethesda, MD) and all others from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM or RPMI containing 10% fetal bovine serum and penicillin-streptomycin.

To establish stable cell lines overexpressing GPNMB, HEK293 cells were transfected with either control vector (pcDNA3.1-V5-His) or this vector containing full-length GPNMB, using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Following selection in medium containing G418 (0.8 mg/mL), individual clones were selected and propagated.

Small interfering RNA (siRNA) was used to inhibit GPNMB expression in SK-Mel-2 cells. Cells were transfected with 50 nmol/L of siGENOME SMART pool reagents (Dharmacon, Inc., Chicago, IL), designed to specifically target GPNMB, or siRNA to thymidylate synthase as a negative control, using the OligofectAMINE transfection reagent (Invitrogen) following the instructions of the manufacturer.

Reverse transcription-PCR and real-time quantitative PCR. Total RNA was isolated using the RNeasy kit with a DNase digestion step (Qiagen, Inc., Valencia CA). Reverse transcription-PCR (RT-PCR) was done using the OneStep RT-PCR kit (Qiagen) as follows. Reverse transcription: 50°C for 45 minutes and 95°C for 15 minutes for one cycle. PCR: 1 minute at 95°C, 1 minute at 50°C, and 2 minutes at 72°C for 30 cycles with final extension for 10 minutes at 72°C. Products were separated on a 2% agarose/0.33% low melting point agarose gel and visualized by ethidium bromide staining. The integrity of each RNA sample was verified via RT-PCR with primers designed to amplify glyceraldehyde-3-phosphate dehydrogenase. The primers used for amplification are as follows (5′-3′):

GPNMB:	Forward-GAATTCAGAGTTAAACCTTGAG
	Reverse-CAGGAATCTGATCTGTTACCAC
MART-1:	Forward-CTGACCCTACAAGATGCCAAGAG
	Reverse-ATCATGCATTGCAACATTTATTGATGGAG
Tyrosinase:	Forward-TTGGCAGATTGTCTGTAGCC
	Reverse-AGGCATTGTGCATGCTGCTT
pMEL-17:	Forward-TATTGAAAGTGCCGAGATCC
	Reverse-TGCAAGGACCACAGCCATC

Real-time quantitative PCR analysis was done with an ABI Prism 7700 Sequence Detection System using TaqMan reagents (PE Applied Biosystems, Foster City, CA). Equal quantities of normalized RNAs were used as a template in PCR reactions for 40 cycles with GPNMB-specific primers to obtain threshold cycle (C_T) values. The primers used for amplification are as follows (5′-3′):

- Forward-TCAATGGAACCTTCAGCCTTA
- Reverse-GAAGGGGTGGGTTTTGAAG
- Probe-TET-CTCACTGTGAAAGCTGCAGCACCAG-TAMRA

Production and purification of recombinant human GPNMB extracellular domain protein. Oligonucleotide primers were designed to amplify the cDNA encoding the GPNMB extracellular domain (GPNMB-ECD) using a human fetal brain cDNA template. The forward primer included an in-frame *Bam*HI site and the reverse primer contained an in-frame *Sal*I restriction site. The primers used for amplification are as follows (5′-3′):

- Forward-GGATCCAAACGATTTCATGATGTGCTGGGCAATGAA
- Reverse-GTCGACCGAGGCTGGGTCTCTGTCAGGAACAGAAAT

The PCR product was cloned into the pCR2.1-Topo vector (Invitrogen). The cDNA insert was verified by sequencing and subcloned into the *Bam*HI/*Xho*I sites of pCEP4 (Invitrogen), which was modified by inserting the murine Igκ secretion signal upstream, and a V5-His tag downstream of the cloning region. This construct was transfected into HEK293 cells using LipofectAMINE 2000 (Invitrogen), and the conditioned medium was tested by immunoblotting for secreted GPNMB-ECD 48 hours posttransfection. Recombinant GPNMB-ECD was purified from the conditioned medium by metal affinity chromatography.

Antibody production. Fully human mAbs directed against GPNMB-ECD were generated using proprietary XenoMouse technology from Abgenix (Fremont, CA) as described (22). Briefly, the human IgG2-bearing XenoMouse strain was immunized twice weekly with 10 μg recombinant GPNMB-ECD. Hybridomas were generated by electrocell fusion. Cell lines were screened for supernatant reactivity with GPNMB-ECD in an ELISA and positive hybridomas were cloned. Antibodies

were purified from hybridoma supernatant fractions by protein-A affinity chromatography.

A human IgG2 antibody isolated from the plasma of myeloma patients was purchased from Fitzgerald Industries International, Inc. (Concord, MA), and used as an isotype-matched control antibody in some experiments.

A rabbit polyclonal antibody to the GPNMB-ECD was generated by immunization with GPNMB-ECD (Rockland Immunochemicals, Gilbertsville, PA) and used for immunoblot analyses.

CR011-vcMMAE production. Synthesis of the activated valine-citrulline linker and auristatin E used in these studies was done following a procedure modified from that previously described (21, 23). Methodology used to couple vcMMAE to CR011 or IgG2 isotype control antibody has been described elsewhere (21). Briefly, CR011 was reduced with Tris-(2-carboxyethylphosphine) at 37°C for 2 hours. The reduction reaction mixture was chilled on ice and treated with prechilled five equivalents of maleimidocaproyl-valine-citrulline-monomethyl auristatin E. After 30 minutes, any free maleimidocaproyl-valine-citrulline-monomethyl auristatin E was quenched by the addition of cysteine (20 mol/mol maleimide) and the conjugate was purified by size-exclusion chromatography, sterile filtered, and stored at −80°C. Antibody-drug conjugates were analyzed for concentration by UV absorbance, aggregation by size-exclusion chromatography, drug/antibody ratio by measuring unconjugated thiols with DTNB after rereduction with DTT, and residual free drug by reverse-phase HPLC. The resulting ADCs used in these studies exceeded 97% monomeric protein. Drug/mAb ratios were calculated to be 2.7 for CR011-vcMMAE and 4.5 for control IgG2-vcMMAE. The level of free drug in all antibody-drug conjugate preparations was <0.5%.

Flow cytometry. GPNMB cell-surface expression was determined by flow cytometry. Approximately 1×10^6 cells were harvested, washed, and incubated with a saturating amount (10 μg/mL) of primary antibody in staining buffer [PBS (pH 7.4), 4% fetal bovine serum, and 0.1% NaN_3] for 30 minutes on ice, followed by washing and staining with R-phycoerythrin-conjugated goat-anti-human secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:100 for 30 minutes on ice. Cells were fixed in 1% paraformaldehyde/PBS and examined on a Becton Dickinson FACSCalibur flow cytometer. Data analysis was done with Becton Dickinson Cell Quest software version 3.3 and the geometric mean fluorescence intensity ratio was determined for each cell type.

Internalization of cell surface bound antibodies was assessed by a modified flow cytometry procedure. In brief, cell suspensions were incubated with 10 μg/mL CR011 for 30 minutes on ice. After washing, incubation was shifted to 37°C for 1 hour to allow internalization of bound antibodies. Cells that remained on ice (total surface bound) or that were incubated at 37°C (internalized) were stained with phycoerythrin-conjugated goat-anti-human secondary antibody at 1:100 for 30 minutes to detect CR011 that had been retained on the cell surface. Labeled cells were analyzed by flow cytometry as described above. The percentage of antibody internalized was determined using the geometric mean ratios and the following formula: % internalized = total surface bound (4°C) − total surface bound (37°C) / total surface bound (4°C) × 100.

Immunoprecipitation and immunoblot analysis. An immunoprecipitation/immonoblotting protocol was used for the analysis of GPNMB protein in cell lysates because CR011 does not work well for immunoblotting. Cells were harvested and lysed on ice for 30 minutes in lysis buffer (1% NP40, 0.15 mol/L NaCl, 0.02 mol/L Tris-HCl, 10% glycerol, and 0.01 mol/L EDTA) supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN). Supernatants were collected and the protein concentration was determined with the BCA Protein Assay kit (Pierce, Rockford, IL). For immunoprecipitation, 2 μg of primary antibody was added to 0.5 to 1 mg of total cell lysates and incubated at 4°C for 3 hours, followed by incubation with protein A-agarose (Amersham Biosciences, Uppsala, Sweden) on ice for 2 hours. The agarose beads were then washed in ice-cold TBST buffer (PBS containing 0.1% Tween 20). Immunoprecipitates were recovered from supernatants after boiling in Laemmli sample buffer and centrifugation.

For immunoblot analysis, total cell lysates (50 μg) or immunoprecipitates were resolved under reducing condition on 4% to 20% Tris-glycine gels (Invitrogen) and transferred to 0.45 μm polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with 3% bovine serum albumin (Sigma, St. Louis, MO) in TBST buffer for 3 hours and probed with rabbit anti-GPNMB polyclonal antibody (1:1,000) for 3 hours. Horseradish peroxidase–conjugated goat anti-rabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch Laboratories) was added and incubated for 30 minutes. The membranes were washed in TBST buffer and subjected to enhanced chemiluminescence (Amersham) following the protocol of the manufacturer.

Immunohistochemistry. For immunohistochemistry, formalin-fixed and paraffin-embedded tissue sample sections derived from human normal or melanoma tissue were stained with CR011. Antigen retrieval was done with partial proteolysis by proteinase K (DakoCytomation, Carpinteria, CA) and endogenous peroxidase activity was quenched in a 3% solution of hydrogen peroxide in methanol.

Tissue sections were first blocked in a solution of 5% bovine serum albumin (Sigma) and 1% goat serum (Jackson ImmunoResearch Laboratories) in PBS for 1 hour, and then incubated with biotinylated CR011 or biotinylated isotype control IgG2 antibody diluted in blocking buffer. After 1 hour, the sections were washed and incubated with horseradish peroxidase–conjugated streptavidin (1:200) for 45 minutes. The washing step was repeated, followed by development of stain using 3,3′-diaminobenzidine reagent (Vector Laboratories, Burlingame, CA). 3,3′-Diaminobenzidine reaction was stopped and the sections were counterstained in hematoxylin, dehydrated, and mounted with Permount.

Clonogenic assays. The *in vitro* growth-inhibitory activity of CR011-vcMMAE was determined by clonogenic assays. Cells were plated in 96-well plates at a subconfluent density and allowed to attach overnight. Cells were then treated with various concentrations of the reagent under analysis and incubated for 4 days at 37°C. Cells were then trypsinized, transferred into six-well plates, and incubated until visible colonies had formed. Colonies were then stained with Giemsa (Sigma) and counted. The surviving cell fractions were calculated based on the ratio of the treated sample and the untreated control. The results were expressed as a percentage of control using GraphPad Prism Version 4 software. The IC_{50} was defined as the concentration resulting in a 50% reduction of colony formation compared with untreated control cultures.

Human melanoma xenograft model. The antitumor effects of the CR011-vcMMAE antibody-drug conjugate were assessed in a human xenograft mouse model. Test animals (5- to 6-week old CD-1 *nu/nu* female athymic mice) were obtained from Harlan Laboratories (Indianapolis, IN) and provided food pellets and water *ad libitum*. *In vivo* studies were carried out with approved institutional animal care and use protocols at Southern Research Institute (Birmingham, AL).

Test animals were implanted s.c. with small fragments of human SK-Mel-2 tumor tissue (30-50 mg) as previously described (24). After tumors became established, mice were treated with test reagents every 4 days for a total of four injections. The effects of treatment were evaluated by tumor measurements. Tumor size (in mg) was calculated using the formula $(W^2 \times L) / 2$. Mice were examined daily and tumor size and body weights were assessed twice weekly. Animals with tumors exceeding 2 g were removed from the study and euthanized.

Results

GPNMB *transcript expression in human melanoma.* A search for genes that are highly expressed in melanoma lead

to our interest in *GPNMB*. Real-time quantitative PCR analysis showed relatively strong expression (C_T < 27) of this gene in five of seven melanoma cell lines and five of five melanoma clinical specimens, and relatively low expression in many other tumor types (Table 1; data not shown).

To extend these results, we examined the expression of *GPNMB* in a panel of 17 melanoma cell lines via semiquantitative RT-PCR (Table 1). This analysis showed that *GPNMB* transcripts are highly expressed in 15 of 17 melanoma cell lines, weakly expressed in 1 of 17 melanoma cell lines (A-375), and not detectable in 1 of 17 melanoma cell lines (LOXIMVI) nor in a renal carcinoma cell line (TK-10). We also examined the expression of known melanocyte/melanoma–associated genes (*MART-1*, *tyrosinase*, *pMEL-17*) in these cells lines and found that most of the melanoma lines coexpressed *GPNMB* along with transcripts for these other genes (Table 1). Both LOXIMVI and TK-10 cell lines, which had undetectable GPNMB

Table 1. *GPNMB* transcript expression in human melanoma samples

RTQ-PCR

Sample type	Sample origin	*GPNMB* expression*
Cell lines		
UACC-62	Met. melanoma	21.2
M14	Met. melanoma, amelanotic	22.2
SK-Mel-5	Met. melanoma, axillary node	22.9
SK-Mel-28	Met. melanoma, skin	24.1
WM-266-4	Met. melanoma, skin	24.5
A-375	Met. melanoma, skin	29.0
LOXIMVI	Met. melanoma, amelanotic	30.9
TK-10	Renal cell carcinoma	40.0
Clinical specimens		
1	Met. melanoma	26.6
2	Melanoma	26.4
3	Melanoma	26.9
4	Met. melanoma	24.1
5	Met. melanoma	25.3

RT-PCR

Cell line	Cell line origin	Expression†			
		GPNMB	*MART-1*	*Tyrosinase*	*pMel-17*
M14	Met. melanoma, amelanotic	+++	+++	+++	+++
SK-Mel-5	Met. melanoma, axillary node	+++	+++	+++	+++
SK-Mel-28	Met. melanoma, skin	+++	+++	+++	+++
WM-266-4	Met. melanoma, skin	+++	+++	+++	+++
SK-Mel-2	Met. melanoma, skin	+++	+++	+++	+++
UACC-257	Met. melanoma	+++	+++	+++	+++
A2058	Met. melanoma, lymph node	+++	+++	+++	+++
G361	Met. melanoma, skin	+++	+++	+++	+++
HT-144	Met. melanoma, skin	+++	+++	+++	+++
MEWO	Met. melanoma, lymph node	+++	+++	+++	+++
SK-Mel-3	Met. melanoma, lymph node	+++	+++	+++	+++
MALME-3M	Met. melanoma	+++	+++	+++	+++
UACC-62	Met. melanoma	+++	+++	+++	–
SK-Mel-24	Met. melanoma, lymph node	+++	–	+++	–
RPMI-7951	Met. melanoma, lymph node	+++	–	+	–
A-375	Met. melanoma, skin	+	–	–	–
LOXIMVI	Met. melanoma, amelanotic	–	–	–	–
TK-10	Renal cell carcinoma	–	–	–	–

Abbreviations: Met., metastatic; RTQ-PCR, real-time quantitative PCR.

*Threshold cycle (C_T) values from real-time quantitative PCR analysis using 40 cycles.

† Based on ethidium bromide staining of PCR product run on agarose gel. +++, strong; +, weak; –, not detectable.

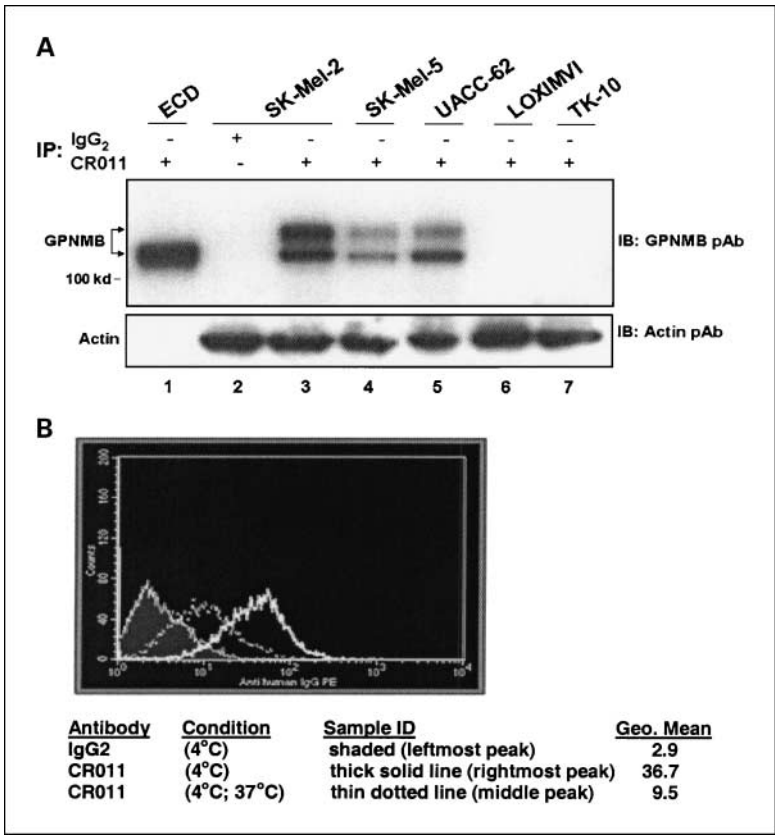

Fig. 1. Detection of GPNMB protein in melanoma cell lines by the CR011 mAb. *A,* purified GPNMB-ECD (*lane 1*) or cell lysates derived from the indicated cell lines (*lanes 2-7*) were immunoprecipitated with CR011 or isotype-matched control IgG2 and then immunoblotted with anti-GPNMB polyclonal antibody (*top*). Lysates were also immunoblotted with an anti-actin antibody (*bottom*). *B,* SK-Mel-2 cells were incubated with CR011 or control IgG2 for 30 minutes at 4°C, after which time unbound antibody was removed by washing. Cells were then kept on ice or incubated at 37°C for 1 hour to facilitate internalization of antibody-antigen complexes. Cell surface – bound CR011 was then detected by flow cytometry using phycoerythrin-conjugated secondary antibody. The geometric fluorescence means are indicated.

expression, also lacked expression of *MART-1*, *tyrosinase*, and *pMEL-17*.

Generation of fully human mAbs to GPNMB. The strong expression of *GPNMB* transcripts and the potential cell surface localization of this protein in human melanoma samples encouraged us to generate mAbs to this protein for potential therapeutic purposes. To this end, we cloned the human GPNMB extracellular domain (amino acids 23-480). Sequencing of the cDNA revealed the presence of an in-frame 36-nucleotide insertion, likely due to alternative splicing at the exon 6/7 boundary, that added an additional 12 amino acids (ATTLKSYDSNTP) after residue 339 of the published GPNMB protein sequence. RT-PCR analysis with primers spanning this region revealed the presence of two products, supporting the likelihood that transcripts with and without the 12-amino-acid addition are authentic (data not shown). The GPNMB-ECD protein was purified from transfected HEK293 cells and used as an immunogen to generate fully human anti-GPNMB mAbs using XenoMouse technology. One particular mAb, designated CR011, exhibited a K_d of 52 nmol/L for purified GPNMB-ECD protein and was selected for additional characterization.

Detection of GPNMB protein in human melanoma samples with anti-GPNMB mAb CR011. Immunoprecipitation of cell lysates with CR011 followed by immunoblotting with a polyclonal antibody that was generated to the GPNMB-ECD revealed the presence of two protein species of ~110 and 130 kDa in melanoma cell lines that express *GPNMB* transcripts (SK-Mel-2, SK-Mel-5, and UACC-62), but not in cell lines devoid of *GPNMB* transcript expression (LOXIMVI, TK-10; Fig. 1A). We hypothesized that the discrepancy in relative mobility between the identified protein species and the predicted molecular weight of GPNMB (63 kDa) was likely due to glycosylation. Our finding that the treatment of SK-Mel-2 lysates with *N*-glycosidase reduced the size of immunoprecipitated GPNMB protein to ~75 kDa supported this hypothesis (data not shown).

We next examined the surface expression of GPNMB protein on a variety of melanoma cell lines by flow cytometry using the CR011 mAb (Table 2). This analysis showed that melanoma cell lines that were positive for *GPNMB* transcript expression also exhibited surface staining with CR011 of at least 1.5-fold above isotype control IgG2 antibody staining. In contrast, two cell lines (LOXIMVI and TK-10) that were devoid of *GPNMB* transcript and protein expression (see Fig. 1A; Table 1) did not express appreciable levels of GPNMB on their cell surface. Flow cytometry results also suggested that a portion of surface-bound CR011 was internalized following incubation at 37°C (Fig. 1B).

Immunohistochemical examination of 58 human melanoma specimens revealed that ~80% were positive for CR011 reactivity, with ~60% of the samples registering an intensity score of ≥2 (out of maximum score of 3). Although staining intensity was sometimes heterogeneous among the individual cells comprising a particular sample, ~60% of the specimens had at least 50% of the cells staining positive with CR011. Among normal tissues examined with CR011, significant

Table 2. GPNMB protein expression on the surface of human melanoma cell lines

Flow cytometry with CR011

Cell line	Sample origin	Geometric mean ratio*
SK-Mel-2	Met. melanoma, skin of thigh	16.5
M14	Met. melanoma, amelanotic	16.1
MEWO	Met. melanoma, lymph node	14.1
WM-266-4	Met. melanoma, skin	13.6
G361	Met. melanoma, skin	8.0
HT-144	Met. melanoma, skin	7.4
UACC-257	Met. melanoma	7.0
RPMI-7951	Met. melanoma, lymph node	6.0
SK-Mel-5	Met. melanoma, axillary node	5.7
UACC-62	Met. melanoma	5.5
A2058	Met. melanoma, lymph node	4.1
SK-Mel-24	Met. melanoma, lymph node	1.9
LOXIMVI	Met. melanoma	1.3
TK-10	Renal clear cell carcinoma	1.1

*Cells were incubated with anti-GPNMB mAb CR011 or isotype control (IgG2) primary antibodies and then with phycoerythrin-conjugated goat anti-human IgG secondary antibody for detection. Geometric mean ratios were determined by dividing the geometric mean fluorescence intensity of cells stained with CR011 by the geometric mean fluorescence intensity of cells stained with the isotype control antibody. LOXIMVI and TK-10 cell lines that do not express detectable *GPNMB* transcripts were used as negative controls.

GPNMB expression was detected in tissue macrophages (Kuppfer cells of the liver, podocytes of the kidney glomerulus, and alveolar macrophages of the lung) and in some cells of the ciliary body of the eye.

In vitro ***growth inhibition of melanoma cell lines with drug-conjugated CR011 (CR011-vcMMAE).*** In preliminary studies, CR011 alone did not inhibit the growth of GPNMB-expressing melanoma cell lines but it was an effective and specific inhibitor of cell growth when combined with a toxin (saporin)–conjugated secondary antibody (data not shown).

To generate a reagent amenable to therapeutic development, CR011 was directly coupled to MMAE, a potent cytotoxic drug. The resulting fully human mAb-drug conjugate was designated CR011-vcMMAE. Coupling of MMAE to CR011 was mediated via a cathepsin-B-cleavable valine-citrulline dipeptide linker, allowing separation of the mAb-drug moieties following intracellular internalization of CR011 drug conjugates. CR011-vcMMAE, which possessed an average of 2.7 MMAE molecules per antibody, was shown to retain cell surface reactivity with GPNMB-expressing SK-Mel-2 melanoma cells via flow cytometry (Table 3).

To examine whether CR011-vcMMAE specifically inhibited the growth of GPNMB-expressing melanoma cells, clonogenic assays were done to assess cell viability following CR011-vcMMAE treatment. Results of these experiments showed that CR011-vcMMAE inhibited the growth of GPNMB-expressing SK-Mel-2 and SK-Mel-5 melanoma cell lines (IC_{50} values of 216 and 300 ng/mL, respectively), but not of GPNMB-negative LOXIMVI and TK-10 cell lines (IC_{50} > 1,000 ng/mL for both cell lines; Table 3). In contrast, MMAE-conjugated isotype control antibody (designated IgG2-vcMMAE) did not inhibit the growth of GPNMB-positive or GPNMB-negative cell lines when used up to 1,000 ng/mL (Table 3), although some growth inhibition was found when higher concentrations were used. Because all cell lines examined were approximately equally sensitive to free MMAE (Table 3), these results suggested that the increased sensitivity of GPNMB-expressing cells to CR011-vcMMAE was not due to an inherent increase in the sensitivity of these cells to the growth-inhibitory effects of MMAE. The specificity of CR011-vcMMAE-mediated growth inhibition was further illustrated by a competition experiment in which unconjugated CR011, but not isotype control antibody, was found to be able to decrease the level of growth inhibition induced by CR011-vcMMAE on SK-Mel-2 cells (data not shown). Additional experiments indicated that CR011-vcMMAE induced G_2-M cell cycle arrest followed by apoptotic cell death in GPNMB-expressing melanoma cells (data not shown).

Growth-inhibitory activity of CR011-vcMMAE is dependent on GPNMB expression. Although the aforementioned experiments strongly suggested that CR011-vcMMAE mediates melanoma cell growth inhibition via interactions with GPNMB, we sought additional experimental proof in this regard. To this end, full-length GPNMB protein was ectopically expressed in HEK293

Table 3. *In vitro* characterization of CR011-vcMMAE

		Cell surface reactivity*		Growth inhibition†			
Cell line	Origin	CR011	CR011-vcMMAE	Free MMAE	CR011	IgG2-vcMMAE	CR011-vcMMAE
SK-Mel-2	Melanoma	16.5	16.0	0.14	>1,000	>1,000	216 ± 61
SK-Mel-5	Melanoma	5.7	Not done	0.10	>1,000	>1,000	300 ± 55
LOXIMVI	Melanoma	1.3	Not done	0.12	>1,000	>1,000	>1,000
TK-10	Renal carcinoma	1.1	1.0	0.13	>1,000	>1,000	>1,000

*Cell surface reactivity was determined by flow cytometry as described in the legend of Table 2. Results are expressed as geometric mean ratios.
† Growth inhibition was determined by clonogenic assays. IC_{50} values represent the mean and SD of three independent assays. IC_{50} values for free MMAE are in nmol/L and IC_{50} values for CR011, IgG2-vcMMAE and CR011-vcMMAE are in ng/mL.

cells that do not endogenously express GPNMB protein. Immunoblot analysis showed GPNMB expression in transfected cells (Fig. 2A), and analysis by flow cytometry showed GPNMB cell surface expression in transfected cells (12-fold increase in geometric mean of GPNMB-transfected cells relative to cells transfected with empty vector; Fig. 2B). When these cells were used for clonogenic assays with CR011-vcMMAE, the IC_{50} for GPNMB-transfected cells was 300 ng/mL, whereas the IC_{50} for cells transfected with empty vector was >1,000 ng/mL. These results indicate that GPNMB cell surface expression is sufficient to mediate sensitivity to the growth-inhibitory effect of CR011-vcMMAE.

Evidence that GPNMB expression is not only sufficient but is in fact required to mediate the growth-inhibitory activity of CR011-vcMMAE was obtained from an experiment in which siRNA was used to specifically repress GPNMB expression in cells (SK-Mel-2) that normally express this protein and are sensitive to growth inhibition by CR011-vcMMAE. Immunoblot analysis showed strong GPNMB expression in SK-Mel-2 cells treated with transfection reagent only ("mock") or control siRNA specific for a gene unrelated to GPNMB, and greatly reduced expression in cells treated with siRNA specific to GPNMB (Fig. 2C). Likewise, analysis by flow cytometry showed strong GPNMB cell surface expression in cells treated with transfection reagent only or control siRNA, and reduced cell surface expression in cells treated with siRNA specific to GPNMB (14-fold decrease in geometric mean of GPNMB-siRNA-treated cells relative to cells transfected with control siRNA following 4 days of siRNA exposure; Fig. 2D). When these cells were used for clonogenic assays with CR011-vcMMAE, the IC_{50} for cells treated with control siRNA was 220 ng/mL whereas that for cells treated with GPNMB-siRNA was 1,000 ng/mL, thus indicating that GPNMB expression was required for efficient growth-inhibition by CR011-vcMMAE.

Inhibition of melanoma tumor growth with CR011-vcMMAE. The antitumor activity of CR011-vcMMAE was examined *in vivo* using a human melanoma SK-Mel-2 xenograft model. As shown in Fig. 3A, the progressive tumor growth that was observed in control animals treated with PBS was inhibited in a dose-proportional fashion in mice treated with CR011-vcMMAE. Significant tumor growth inhibition was evident at the lowest concentration of CR011-vcMMAE examined (0.313 mg/kg/dose) and complete tumor regressions were observed in most animals treated with CR011-vcMMAE at concentrations of ≥1.25 mg/kg. Most tumors that had regressed following CR011-vcMMAE treatment did not regrow during a posttreatment observation period of 200 days.

In a separate xenograft experiment, the effect of unconjugated CR011 mAb and free MMAE was examined (Fig. 3B).

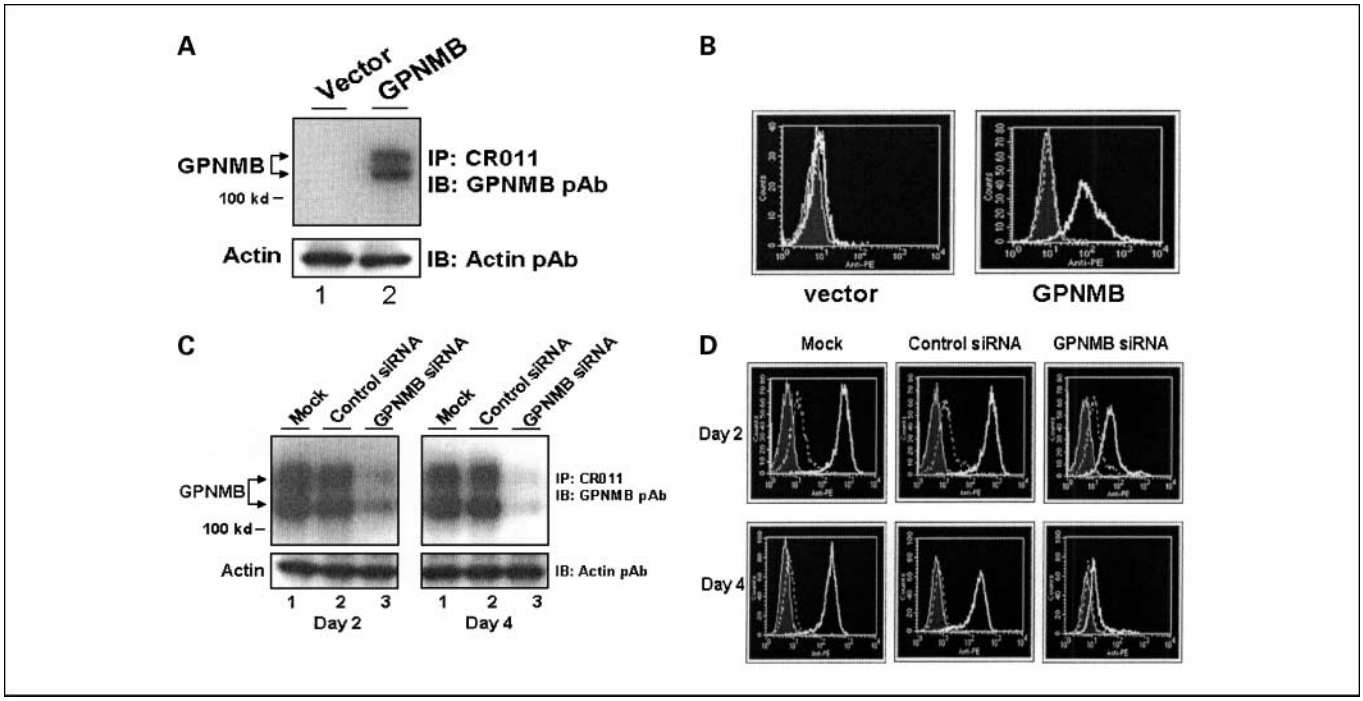

Fig. 2. Generation and characterization of cells with ectopic overexpression or repressed expression of GPNMB. *A,* cell lysates from HEK293 cells (GPNMB-negative) transfected with empty vector (*lane 1*) or GPNMB vector (*lane 2*) were immunoprecipitated with CR011 and then immunoblotted with anti-GPNMB polyclonal antibody. Lysates were also immunoblotted with an anti-actin antibody (*bottom*). *B,* HEK293 cells transfected with empty vector (*left*) or GPNMB vector (*right*) were analyzed for cell-surface expression of GPNMB by flow cytometry. Primary antibodies were as follows: no primary (*shaded peak*); isotype control IgG2 (*dotted line*); CR011 (*thick solid line*). *C,* cell lysates from SK-Mel-2 cells exposed to no siRNA (*lane 1*), negative control siRNA (*lane 2*), or siRNA to GPNMB (*lane 3*) were immunoprecipitated with CR011 and then immunoblotted with anti-GPNMB polyclonal antibody. Left, cells examined 2 days after exposure to siRNA. Right, cells examined 4 days after exposure to siRNA. Lysates were also immunoblotted with an anti-actin antibody (*bottom*). *D,* SK-Mel-2 cells exposed to no siRNA (*left*), negative control siRNA (*middle*), or siRNA to GPNMB (*right*) were analyzed for cell surface expression of GPNMB by flow cytometry. Primary antibodies were as follows: no primary (*shaded peak*); isotype control IgG2 (*dotted line*); CR011 (*thick solid line*).

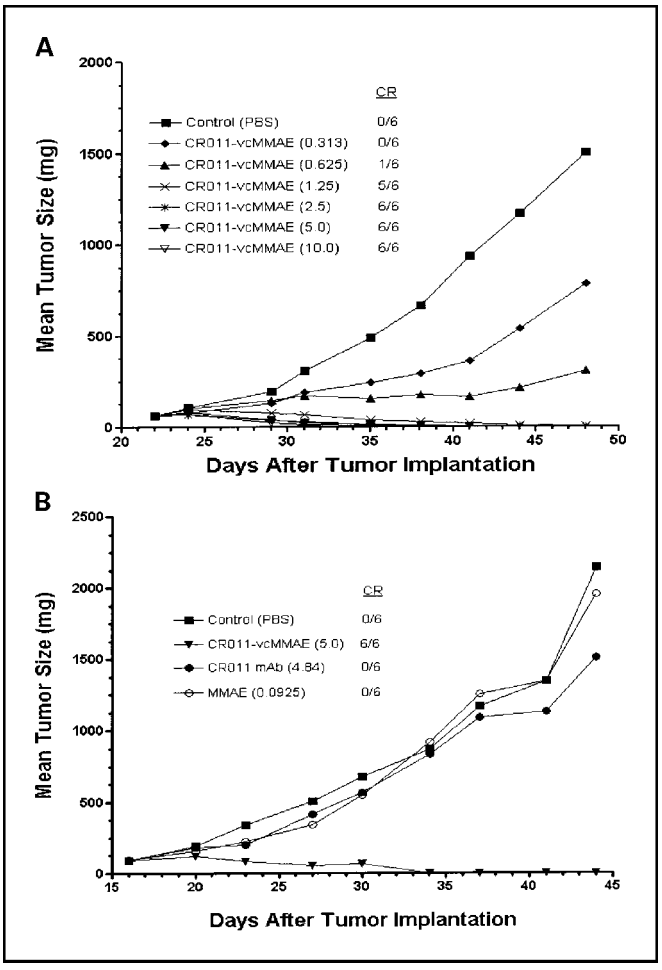

Fig. 3. Tumor growth inhibition by CR011-vcMMAE. *A,* athymic mice were implanted s.c. with fragments of SK-Mel-2 tumors. After tumors became established (day 22), animals were administered CR011-vcMMAE at the indicated doses (in mg/kg/dose) or PBS every 4 days for a total of four treatments. The number of animals in each group that showed complete tumor regression (*CR*) is indicated. Data from groups receiving 1.25, 2.5, 5.0, and 10 mg/kg/dose overlap on the *X* axis. *B,* experiment was done essentially as described in (*A*) and was designed to examine the effect of unconjugated CR011 mAb and free MMAE on tumor growth relative to CR011-vcMMAE administered at 5.0 mg/kg/dose. Treatment was initiated on day 16.

The results of this experiment showed that neither unconjugated CR011 mAb nor free MMAE used at concentrations equivalent to that of CR011-vcMMAE dosed at 5.0 mg/kg inhibited tumor growth. In contrast, CR011-vcMMAE (5.0 mg/kg/dose) again exhibited significant tumor growth inhibition and complete tumor regressions. Tumor regressions were also found when CR011-vcMMAE was administered to mice harboring xenografts derived from another GPNMB-positive human melanoma cell line (SK-Mel-5; data not shown).

No toxic deaths of treated animals were observed in the xenograft experiments nor were there indications of abnormal behavior or other nonspecific signs of drug toxicity. Moreover, twice weekly body weight measurements did not show significant weight loss or inhibition of weight gain as a consequence of CR011-vcMMAE administration. Subsequent studies indicated that the threshold for overt toxicity of CR011-vcMMAE in mice involves doses that are >5-fold higher than those used in the present study. A preliminary pharmacokinetic analysis in mice shows the antibody component of CR011-vcMMAE to have an elimination half-life of ~10 days (data not shown).

Discussion

Antibodies directed toward tumor-associated antigens are emerging as potent clinical cancer therapeutics (9, 25). In the present investigation, we found *GPNMB* transcript expression in the majority of metastatic melanoma cell lines and clinical samples examined. This observation, together with the facts that GPNMB possesses a signal peptide, is predicted to be a transmembrane protein, and is not strongly expressed in most normal tissues (16), prompted us to generate fully human anti-GPNMB mAbs for potential therapeutic use. The in-depth characterization of one such mAb (CR011) showed that this mAb could immunoprecipitate GPNMB protein from the lysates of *GPNMB* transcript–positive melanoma cell lines. This mAb also stained the cell surface of most metastatic melanoma cell lines examined, and at least a portion of the resulting antibody-antigen complex was apparently internalized. Finally, CR011 reacted with the majority of metastatic melanoma clinical samples examined by immunohistochemistry and revealed a restricted normal tissue distribution. These results encouraged us to evaluate CR011 as a potential therapeutic for melanoma.

Because unconjugated CR011 did not by itself inhibit melanoma cell growth, we generated a reagent, CR011-vcMMAE, which combines the tumor-targeting specificity of a mAb (CR011) and the cytotoxic activity of a potent antimitotic compound (MMAE). Our findings show that CR011-vcMMAE inhibits the growth of GPNMB-expressing melanoma cells *in vitro* an *in vivo*.

Our *in vitro* experiments indicate that CR011-vcMMAE is selectively active on GPNMB-expressing cells. Naturally occurring GPNMB-negative tumor cells were not growth inhibited by CR011-vcMMAE at concentrations (e.g., 200-300 ng/mL) that effectively inhibited the growth of GPNMB-positive melanoma cells. A control reagent, IgG2-vcMMAE, did not inhibit the growth of GPNMB-positive or GPNMB-negative cell lines at concentrations <1,000 ng/mL. Moreover, the manipulation of GPNMB expression by gene transfection and siRNA in culture systems showed that GPNMB expression is both necessary and sufficient for potent CR011-vcMMAE-mediated growth inhibition.

The animal experiments reported herein show that CR011-vcMMAE effectively inhibits and eradicates SK-Mel-2 human melanoma xenograft growth even when administered at relatively low doses (e.g., 1.25 mg/kg/dose). In contrast, the biologically active components of this immunoconjugate, namely the CR011 mAb and the free MMAE drug, do not inhibit tumor growth when separately administered at doses equivalent to 5.0 mg/kg of CR011-vcMMAE.

Although no overt CR011-vcMMAE toxicity was observed in the animal experiments reported here, CR011 does not cross-react with murine GPNMB (data not shown), thus limiting the usefulness of this animal species for toxicity evaluation. Examination of human samples indicates that GPNMB is not strongly expressed in many normal tissues (ref. 16; this study). Furthermore, the intracellular domain of GPNMB contains a dileucine motif that may play a role in the intracellular retention of this protein in some instances (26). Thus, in normal cells that may express GPNMB, it remains to be determined whether any GPNMB protein is present at the cell surface. Also, although the effect of CR011-vcMMAE on normal nonproliferating cells is unknown, the possibility remains that this reagent would be less cytotoxic to these cells than to those that are rapidly dividing due to the nature of the MMAE drug. A preliminary experiment in monkeys indicates that CR011-vcMMAE is well tolerated at multiple does that show antitumor activity in mice (data not shown). These findings support the possibility that a therapeutic index can be established for CR011-vcMMAE in humans. Although the CR011 mAb is fully human and thus should not elicit an immune response, the potential immunogenicity of the CR011-vcMMAE reagent in humans remains to be determined.

Although most of the data available in the literature supports a potential functional role for GPNMB in malignant progression (see Introduction), GPNMB was originally identified as a gene that was more highly expressed in a melanoma cell line (MV1) with low metastatic potential in nude mice compared with its more metastatic counterpart (MV3; ref. 12). MV1 and MV3 represent cell lines that were derived from the same metastatic melanoma lesion, which differ in their ability to generate metastasis in nude mice. GPNMB was also found to be poorly expressed in another melanoma cell line (BLM) with high metastatic potential in nude mice. However, it should be mentioned that MV3 and BLM cell lines do not express melanocyte/melanoma–associated genes, such as *pMEL-17*, *tyrosinase*, and *MART-1* (27, 28), and thus it is not surprising that these two lines also do not express GPNMB because we have shown that GPNMB expression is generally correlated with that of these melanocyte/melanoma–associated genes (see Table 1). Our transcript expression data shows that *GPNMB*, *pMEL-17*, *tyrosinase*, and *MART-1* are coexpressed in the majority of melanoma cell lines tested, most of which were isolated from metastasis. Moreover, we have found by flow cytometry using the CR011 mAb that GPNMB protein is expressed on the cell surface of most of these melanoma cell lines (see Table 2), and by immunohistochemical examination that ~80% of metastatic melanoma specimens stain positive with this mAb. Our results are consistent with several literature reports that show that the majority of metastatic melanoma specimens retain expression of melanocyte/melanoma–associated antigens, such as pMEL-17, tyrosinase, and MART-1 (29–31). However, a minority of metastatic melanoma samples apparently lack expression of these antigens as well as GPNMB, and the MV3/BLM (12) and LOXIMVI (this report) cell lines may be representative of such clinical specimens. The level of tumor-associated GPNMB expression is an important variable to consider in the future clinical development of CR011-vcMMAE.

Acknowledgments

We thank Donald Dykes and Murray Stackhouse at Southern Research Institute for their help and expertise in performing the xenograft studies.

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