Abstract

**Purpose:** The goal of this research was to harness a monoclonal antibody (mAb) discovery platform to identify cell-surface antigens highly expressed on cancer and develop, through Fc optimization, potent mAb therapies toward these tumor-specific antigens.

**Experimental Design:** Fifty independent mAbs targeting the cell-surface immunoregulatory B7-H3 protein were obtained through independent intact cell-based immunizations using human tissue progenitor cells, cancer cell lines, or cell lines displaying cancer stem cell properties. Binding studies revealed this natively reactive B7-H3 mAb panel to bind a range of independent B7-H3 epitopes. Immunohistochemical analyses showed that a subset displayed strong reactivity to a broad range of human cancers while exhibiting limited binding to normal human tissues. A B7-H3 mAb displaying exquisite tumor/normal differential binding was selected for humanization and incorporation of an Fc domain modified to enhance effector-mediated antitumor function via increased affinity for the activating receptor CD16A and decreased binding to the inhibitory receptor CD32B.

**Results:** MGA271, the resulting engineered anti–B7-H3 mAb, mediates potent antibody-dependent cellular cytotoxicity against a broad range of tumor cell types. Furthermore, in human CD16A-bearing transgenic mice, MGA271 exhibited potent antitumor activity in B7-H3–expressing xenograft models of renal cell and bladder carcinoma. Toxicology studies carried out in cynomolgus monkeys revealed no significant test article-related safety findings.

**Conclusions:** This data supports evaluation of MGA271 clinical utility in B7-H3–expressing cancer, while validating a combination of a nontarget biased approach of intact cell immunizations and immunohistochemistry to identify novel cancer antigens with Fc-based mAb engineering to enable potent antitumor activity. *Clin Cancer Res; 18(14); 3834–45. ©2012 AACR.*

Introduction

Antigens that are tumor specific or overexpressed on cancer cells represent opportunities for development of target-specific antibody-based therapeutics with a range of possible therapeutic modalities. For example, unmodified IgG1 monoclonal antibodies (mAb) directed to the EGF receptor (EGFR) family, neutralize tumor-promoting activities of such molecules through antiproliferative and tumor-}

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Development of Fc-Enhanced Anti–B7-H3 Monoclonal Antibody

Translational Relevance
Using a monoclonal antibody (mAb)–driven process to identify cell-surface antigens highly expressed in cancer, we discovered a panel of mAbs against B7-H3, a member of the B7 family of immunomodulatory molecules. To translate this discovery for clinical evaluation, we developed MGA271, a humanized B7-H3 mAb displaying broad tumor reactivity but limited normal tissue binding that incorporates Fc-domain modifications designed to enhance antitumor effector-mediated function. The rationale that potent mAb-mediated effector functions will be clinically beneficial is supported by comparison of clinical outcomes of mAb therapies with natural polymorphisms of FcRs, which revealed more favorable outcome for patients homozygous for the higher affinity alleles of the activating receptors CD16A and CD32A. We hypothesize that the enhanced antitumor effects of MGA271 in preclinical studies, combined with its favorable safety profile in non-human primates, as shown in this article, will translate into potent antitumor activity toward B7-H3–positive cancers in the clinic.

responses were observed in patients who were homozygous for the higher affinity alleles of FcγR IIA (CD32A) and, in particular, FcγR IIIA (CD16A). These therapeutic mAbs include rituximab for treatment of follicular lymphoma (4, 5), trastuzumab for treatment of metastatic breast cancer (6), and, albeit controversial, cetuximab for treatment of metastatic colorectal cancer (mCRC; ref. 7). These results support the notion that potent immune-mediated effector functions can be clinically beneficial, and mAbs with enhanced Fc-mediated activity may favor not only patients with lower affinity allelic variants for FcγRs but also those individuals who are homozygous for the higher binding alleles. This strategy can be exploited for cell-surface cancer targets irrespective of their causality in the initiation or progression of the tumor.

To identify cell-surface cancer antigens suitable for mAb-based targeting, we have characterized mAbs generated in a target-unbiased fashion from intact cell immunizations of serum-free adapted tissue progenitor cells or cancer cell lines, including those exhibiting cancer stem cell properties (8, 9). Immunohistochemical profiling of these mAbs reveals those displaying differential expression on human cancer tissues compared with normal tissue, whereas biochemical analyses identify the cell-surface cancer antigen recognized (10). Here we describe characterization of a panel of 50 independent mAbs identified from this approach that recognize the cell-surface protein B7-H3 (CD276), a member of the B7 family of immune regulators (11). The limited normal tissue binding and broad tumor reactivity exhibited by specific B7-H3 mAbs prompted development of MGA271, a fully humanized anti-B7H3 mAb bearing an engineered Fc domain optimized to enhance antitumor effector–mediated function through combination of enhanced binding to the activating receptor CD16A and reduced binding to the inhibitory receptor CD32B. To determine the clinical potential of MGA271, antitumor activity was evaluated in a series of murine xenograft models and its toxicology profile evaluated in non-human primates.

Materials and Methods

Cell lines
Renal (A498, 786-o, and ACHN), prostate (LnCap), lung (SK-MES-1), breast (MDA-MB-468), bladder (SW780 and HT-1197), melanoma (UACC-62) cancer cell lines, and Raji B-cell lymphoma were obtained from American Type Culture Collection and cultured according to recommended protocol for fewer than 20 passages.

Immunohistochemistry

OCT-embedded, frozen tissues, and positive control B7-H3–expressing Caki2 and Hs700T cells were sectioned at 7 μm. Following drying, sections were incubated in 4°C acetone, air dried, then processed in a Dako Autostainer. Formalin-fixed paraffin-embedded (FFPE) tissue microarray sections were deparaaffinized, rehydrated, then processed in a Dako Autostainer. Endogenous peroxidase activity was quenched with 3% H2O2, then nonspecific binding sites were blocked with 5% normal goat serum. Primary mAbs (BRCA84D and BRCA69D) were detected using EnVision horseradish peroxidase (HRP) anti-mouse polymer (Dako) in conjunction with 3,3’-Diaminobenzidine (Sigma-Aldrich). Slides were counterstained with hematoxylin.

Protein engineering

cBRCA84D was generated by fusing the BRCA84D VL and VH coding sequences to human ε-Kappa or human gamma 1 constant region cDNA, respectively. To construct hBRCA84D, humanized BRCA84D VL (hBRCA84D VL) and BRCA84D VH (hBRCA84D VH) amino acid sequences were designed using the CDR sequences from the mouse mAb BRCA84D and framework sequences from human germline V-kappa or VH segment, respectively. The hBRCA84D VL and hBRCA84D VH coding sequences were synthesized de novo, fused to the human C-Kappa or human gamma 1 constant region cDNA, respectively. Single mutations, L46A in VL and A93G in VH, were introduced by site-directed mutagenesis to optimize the binding affinity for B7-H3.

MGA271 was generated from hBRCA84D by exchanging its Fc domain for MGFlc0264 (L235V, F243L, R292P, Y300L, and P396L). hBRCA84D-aglycosyl was generated from hBRCA84D by mutagenesis at the N-glycosylation site of the WT Fc domain. Antibodies were produced in stably transfected Chinese hamster ovary cells.

ADCC
Peripheral blood mononuclear cells (PBMC) were isolated from healthy human donor blood (Ficoll-Paque Plus; GE Healthcare). Target cells, effector cells, and antibody
were incubated overnight in Dulbecco’s modified Eagle’s medium/F-12 containing 5% FBS. PBMC effector cells were added at ratios of 25:1 to 30:1. LDH release (Promega Corp.) was measured after overnight incubation. Cytotoxicity (%) = (experimental cell lysis – antibody-independent cell cytolysis)/(maximum target lysis – spontaneous target lysis) × 100. Fcγ receptor genotypes were determined by sequencing PCR-amplified DNA. To evaluate the ability of murine B7-H3 mAb to support ADCC, FITCylated anti–B7-H3 was mixed with a bispecific DART (Dual Affinity ReTargeting; CD16–CD16) comprising specificity of human CD16 and fluorescein isothiocyanate (FITC) and incubated with cancer target cells together with resting human PBMC effector cells (E:T = 30:1).

Mass spectrometry

GB8 antigen was immunoprecipitated from A498 cell membranes using biotinylated antibody and streptavidin-coated resin (Pierce). After washing, antigens were eluted with low pH buffer and concentrated using Strataclean membranes using biotinylated antibody and streptavidin. GB8 antigen was immunoprecipitated from A498 cell membranes using biotinylated antibody and streptavidin-coated resin (Pierce). After washing, antigens were eluted with low pH buffer and concentrated using Strataclean membranes using biotinylated antibody and streptavidin. GB8 antigen was immunoprecipitated from A498 cell membranes using biotinylated antibody and streptavidin-coated resin (Pierce). After washing, antigens were eluted with low pH buffer and concentrated using Strataclean membranes using biotinylated antibody and streptavidin. GB8 antigen was immunoprecipitated from A498 cell membranes using biotinylated antibody and streptavidin-coated resin (Pierce). After washing, antigens were eluted with low pH buffer and concentrated using Strataclean membranes using biotinylated antibody and streptavidin.

Capture ELISA

A MaxiSorp ELISA plate (Nalge Nunc Intl.) was coated with soluble human B7-H3(4Ig)-His (0.3 µg/mL) in BupH bicarbonate buffer (Thermo Fisher Scientific) overnight at 4°C. The plate was blocked with PBS containing 0.5% bovine serum albumin (BSA) and 0.1% Tween-20 (PBST/BSA) for 30 minutes. Antibodies were diluted in PBST/BSA and applied to the ELISA plate for 1 hour. Following wash with PBST, HRP-conjugated goat anti-mouse IgG (H+L; dilution 1:10,000 in PBST/BSA; Jackson ImmunoResearch) was added for 1 hour, the plate washed and developed with 80 µL/well of TMB peroxidase substrate and terminated with 40 µL/well 1% H2SO4. Absorbance at 450 nm (A450) was determined and data analyzed using GraphPad Prism 5 software.

SPR analysis of human B7-H3 binding to selected mAbs

Binding of the B7-H3 mAb panel to human B7-H3 was analyzed by surface plasmon resonance (SPR) in a BIAcore 3000 biosensor (Biacore AB) as previously described (12, 13). mAbs were captured on goat anti-mouse F(ab’)2 fragment (Jackson ImmunoResearch) coated CM-5 sensor chips and binding curves obtained following injection of human B7-H3(4Ig)-His (R&D Systems). Experimental binding curves were also generated following injection of BRCA8A4D and its humanized forms to both human and cynomolgus monkey B7-H3 on the CM-5 sensor chip. Data were analyzed using BIAevaluation 4.0 software. Kinetic constants, kₐ and kₐ, describing the binding of first arm of antibody to immobilized antigen were estimated by global fitting analysis of the association/dissociation curves to the bivalent analyte interaction model. The equilibrium dissociation constant (Kₐ) was calculated as Kₐ = kₐ/kₐ.

In vivo efficacy

All mouse experiments were carried out under protocols approved by the MacroGenics Institutional Animal Care and Use Committee (IACUC). mCD16−/− hCD16A+ RAG2−/− mice (mCD16 knockout mice expressing the hCD16A-158F transgene consistent with the distribution of CD16A in human tissues) were bred at MacroGenics. Tumor cells (5 × 10⁶ per mouse) in PBS + Matrigel were implanted subcutaneously and antibodies administered intravenously weekly, beginning approximately 1 week following tumor implantation or after tumors of approximately 200 to 300 mm³ had been allowed to form. Tumor sizes were monitored twice weekly by orthogonal measurements with electronic calipers. Statistical differences in tumor sizes were assessed by two-way ANOVAs and Bonferroni posttest analyses (GraphPad Prism 5.02).

Pharmacokinetics/toxicology studies in cynomolgus monkeys

Cynomolgus monkey experiments were conducted at SNBL USA, which adheres to the regulations outlined in the USDA Animal Welfare Act and the conditions specified in the Guide for the Care and Use of Laboratory Animals. The study protocols were approved by the Testing Facility IACUC. A single-dose study was conducted with 24 cynomolgus monkeys randomized into 4 groups (3/gender/group) receiving vehicle control or MGA271 at 1, 30, or 150 mg/kg by 60-minute intravenous infusion. Terminal group animals (2/gender/group) were necropsied 7 days following dose administration. Recovery group animals (1/gender/group) were euthanized 40 days following dose administration for necropsies. A repeat-dose study was conducted with 52 cynomolgus monkeys randomized into 5 groups receiving vehicle control or MGA271 at 1, 10, 30, or 150 mg/kg by 60-minute intravenous infusion. Terminal group animals (4/gender/group for vehicle control; 3/gender/group for MGA271) were necropsied 7 days following the final dose administration. Recovery group animals (2/gender/group) were necropsied 70 days following the final dose.

Results

Identification of a panel of B7-H3 mAbs

Through a series of intact cell immunizations, we have obtained more than 1,500 mAbs reactive with antigens expressed on cancer cells. Antibodies discovered include those recognizing common cancer antigens such as EGFR, HER2, and CEACAM5 and those binding unique cancer antigens such as RAAG12 (10). A subset of antibodies that displayed strong normal/tumor differential by immunohistochemistry (Fig. 1A) exhibited a distinct binding fingerprint across a panel of cancer cell lines, suggesting reactivity to an alternate cancer-associated
Development of Fc-Enhanced Anti–B7-H3 Monoclonal Antibody

Among the panel of B7-H3 mAbs initially screened on frozen tissue specimens, BRCA84D exhibited the least reactivity to normal tissues, while retaining strong reactivity with prostate, breast, colon, lung, gastric, and renal cancers (Supplementary Fig. S1A). As a further test of the differential reactivity of BRCA84D, analysis of reactivity toward lung squamous carcinoma compared with normal adjacent lung tissue from the same patient specimen confirmed that BRCA84D exhibited strong reactivity toward lung squamous carcinoma compared with normal adjacent lung tissue from the same patient specimen (Table 1).

Selection, humanization, and Fc-optimization of BRCA84D as a clinical candidate for B7-H3–directed interventions

Figure 1. Tumor-specific panel of mAbs are reactive with human B7-H3. A, anti–B7-H3 mAb TES7 exhibits strong differential reactivity to multiple solid tumor tissues compared with normal tissues. No positive staining was noted in human normal pancreas, lung, liver, kidney, and heart. The very weak staining in the epithelium of the crypt of the colon was consistent with a nonspecific staining pattern. B, tandem mass spectrometry analysis of protein immunoprecipitated by GB8 mAb from A498 membrane lysate identifies B7-H3 as the potential antigen. TOF peptide matches are highlighted in bold. C, confirmation of reactivity of GB8 for human B7-H3 by ELISA using recombinant human B7-H3. D, anti–B7-H3 mAb BRCA84D exhibits strong differential reactivity to lung squamous carcinoma compared with normal adjacent lung tissue from the same patient specimen.

antigen. Tandem mass spectrometry of protein immunoprecipitated from A498 renal cell carcinoma cell membranes by one mAb in the set, GB8, yielded several peptides corresponding to B7-H3 (Fig. 1B). A recombinant B7-H3–based ELISA assay confirmed that GB8 (Fig. 1C), as well as the other mAbs in the subset (data not shown), were reactive with B7-H3. The tumor-specific reactivity of this set of mAbs prompted screening of the larger panel for additional mAbs and led to the identification of 50 B7-H3–reactive mAbs. A subset of the B7-H3–reactive mAbs, which recognized nonoverlapping epitopes on B7-H3 and displayed a range of binding affinities, is shown in Table 1.

To determine the ability of the B7-H3 mAb panel to support immune effector cell-based interventions, mAbs were tested for ADCC activity. This was assessed in a cell-based cytotoxicity assay using a FITCylated panel of B7-H3 mAbs, together with a bispecific DART molecule recognizing FITC and CD16A (CD16AxFITC DART; ref. 14). The CD16AxFITC DART molecule engages the targeting mAb via the FITC specificity and engages immune effector cells through the anti-CD16 arm, thus bringing together target and effector cells. This strategy obviates the lack of effector function intrinsic in the majority of the murine B7-H3 mAbs. All but one of the B7-H3 mAbs tested efficiently redirected immune-mediated killing of B7-H3–positive A498 renal cell carcinoma cells (Table 1).
cross-reactivity, BRCA84D was chosen for further development. To generate MGA271, the coding sequences of the murine IgG1 mAb variable light and heavy chain genes were humanized and then fused in-frame with MGFc0264, an optimized human IgG1 Fc domain containing 5 amino acid changes that impart increased affinity for both alleles of the human activating FcR, CD16A, and decreased affinity for the inhibitory FcR, CD32B (12). Control humanized BRCA84D mAbs containing either the wild-type human IgG1 Fc domain (RES240) or a mutated human IgG1 Fc domain that lacks FcR binding (RES240-aglycosyl) were also constructed. The recombinant mAbs retained the binding affinity of the parental BRCA84D mAb for both human and cynomolgus monkey B7-H3 (Supplementary Table S1). Importantly, a survey analysis confirmed the restricted reactivity of MGA271 with a broad set of normal tissues. Membrane staining was limited to basal squamous epithelium of the skin (1–2+, occasional) when tested at the optimal staining concentration. Additional membranous staining (1+, rare) of squamous epithelium of the esophagus was observed when stained at 5 times the optimal concentration. Cytoplasmic staining (1+, rare to occasional) was observed in various epithelium and stroma at the optimal concentration and was modestly increased in intensity and frequency at 5 times the staining concentration (Supplementary Table S2). Because of the possible immune regulatory capacity of B7-H3, reactivity of MGA271 in lymphatic tissues was also examined. No reactivity of MGA271 with lymph node and spleen tissues was observed (Supplementary Fig. S1B).

B7-H3 is highly expressed in multiple cancers with high penetrance

An expanded panel of FFPE tumor tissues was screened to confirm the expression level and penetrance of B7-H3 in cancer. Because BRCA84D does not perform adequately on paraffin-embedded tissues, we employed BRCA69D, another B7-H3 mAb from our panel that performs appropriately for these tissues. We identified 28 distinct tumor types, represented by a total of 649 paraffin blocks, for testing. All tissues were stained with the parental BRCA69D mAb, with additional staining using the optimized MGA271 mAb. The staining patterns of the parental BRCA69D mAb were highly concordant with those of MGA271, allowing for the robust assessment of B7-H3 expression (Supplementary Table S3).

Table 1. Select panel of tumor-reactive mAbs recognizing nonoverlapping epitopes on human B7-H3

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Input cell line</th>
<th>Epitope group</th>
<th>Affinity (KD, nmol/L)</th>
<th>In Vitro redirected killing* (% cytotoxicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA69D</td>
<td>IgG1</td>
<td>MCF-7</td>
<td>A</td>
<td>2.4</td>
<td>82</td>
</tr>
<tr>
<td>BRCA84D</td>
<td>IgG1</td>
<td>MCF-7</td>
<td>B</td>
<td>28.3</td>
<td>72</td>
</tr>
<tr>
<td>GB8</td>
<td>IgG1</td>
<td>Fetal gall bladder progenitor</td>
<td>C</td>
<td>22.7</td>
<td>38</td>
</tr>
<tr>
<td>OVCA64</td>
<td>IgG1</td>
<td>Ovarian CSCs</td>
<td>D</td>
<td>0.8</td>
<td>72</td>
</tr>
<tr>
<td>PRCA157</td>
<td>IgG1</td>
<td>H460</td>
<td>E</td>
<td>15.0</td>
<td>59</td>
</tr>
<tr>
<td>SG27</td>
<td>IgG2b</td>
<td>Fetal sweat gland progenitor</td>
<td>F</td>
<td>263.2</td>
<td>15</td>
</tr>
<tr>
<td>TES7</td>
<td>IgG1</td>
<td>Fetal testes progenitor</td>
<td>G</td>
<td>45.3</td>
<td>83</td>
</tr>
</tbody>
</table>

*Redirected killing of A498 renal cell carcinoma tumor cells using FITCylated murine anti–B7-H3 mAbs together with bispecific (CD16×FIC) DART in the presence of resting human PBMC effector cells (E:T = 30:1).

Table 2. Summary of immunohistochemical staining of FFPE tumor specimens with anti–B7-H3 mAb BRCA69D to evaluate B7-H3 expression across a broad range of cancer types

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Type</th>
<th>Positive/total</th>
<th>%Positive</th>
<th>Moderate to high staining (2+ or greater)</th>
<th>%Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>Primary</td>
<td>48/51</td>
<td>94</td>
<td>25/51</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Metastatic</td>
<td>18/19</td>
<td>95</td>
<td>7/19</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>66/70</td>
<td>94</td>
<td>32/70</td>
<td>46</td>
</tr>
<tr>
<td>Kidney cancer</td>
<td>Primary</td>
<td>77/78</td>
<td>99</td>
<td>75/78</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Metastatic</td>
<td>88/99</td>
<td>89</td>
<td>51/99</td>
<td>52</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Primary</td>
<td>69/78</td>
<td>88</td>
<td>45/78</td>
<td>58</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Primary</td>
<td>100/115</td>
<td>87</td>
<td>100/115</td>
<td>87</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Primary</td>
<td>76/90</td>
<td>84</td>
<td>74/90</td>
<td>82</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Primary</td>
<td>39/52</td>
<td>75</td>
<td>19/52</td>
<td>37</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Metastatic</td>
<td>4/8</td>
<td>50</td>
<td>2/8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>43/60</td>
<td>72</td>
<td>21/60</td>
<td>35</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>Primary</td>
<td>12/75</td>
<td>16</td>
<td>6/75</td>
<td>8</td>
</tr>
</tbody>
</table>
Development of Fc-Enhanced Anti–B7-H3 Monoclonal Antibody

on paraffin-embedded tissues. The tumor reactivity against a panel of solid primary and metastatic tumor tissue samples is summarized in Table 2. Representative examples of low (1+), moderate (2+), and high (3+) B7-H3 expression levels in cancer tissues, as detected by BRCA69D, are shown in Fig. 2A. In kidney cancer, substantial expression was observed not only in the epithelial component of the tumor but also in the surrounding tumor vasculature (Fig. 2B).

**Humanized/Fc-optimized BRCA84D (MGA271) mediates potent ADCC in vitro**

To confirm retention of ADCC activity and determine the effect of the MGFct0264, MGA271 was compared with chimeric BRCA84D (chBRCA84D, WT Fc domain), humanized BRCA84D (RES240, WT Fc domain), and aglycosylated humanized BRCA84D (RES240-aglycosyl, inactivated Fc domain) using human PBMC donors representing the 3 CD16A genotypes (low-affinity 158F homozygous, high-affinity 158V homozygous, and 158F/V heterozygous). As shown in Fig. 3A and B, RES240 mediates ADCC in vitro against B7-H3–expressing A498 renal cell carcinoma cells with a potency equivalent to chimeric BRCA84D, indicating that humanization did not alter ADCC function. Importantly, MGA271 exhibited enhanced potency in the ADCC assay relative to RES240. The enhanced ADCC potency of MGA271 was evident with PBMCs from all 3 CD16A donor genotypes (Fig. 3A–C), with the greatest enhancement observed for the homozygous low-binding 158F allele of CD16A, consistent with the enhanced FcγR-binding characteristic of MGA271 Fc domain for this CD16A allele. As expected, RES240-aglycosyl did not mediate ADCC (Fig. 3C), indicating that the cytotoxic activity observed with MGA271, RES240, and chBRCA84D is a function of Fc/FcγR interactions. The MGFct0264 Fc of MGA271 retained binding to cynomolgus monkey FcγR (13) and translated into functional engagement, as shown by the ability of MGA271 to mediate ADCC against A498 cells with cynomolgus monkey PBMCs (Fig. 3D).

MGA271-mediated ADCC activity was determined for a panel of tumor cell lines displaying a range of B7-H3 cell-surface expression and representing multiple cancer types, including kidney, prostate, lung, breast, and bladder carcinoma as well as melanoma (Supplementary Fig. S2). All cancer cell lines that displayed detectable levels of B7-H3 expression exhibited sensitivity to MGA271-mediated ADCC (Fig. 4). Consistent with the enhanced ADCC observed with MGA271 against A498 cells, MGA271 exhibited enhanced ADCC potency relative to RES240 against all
B7-H3–expressing tumor cell lines examined (data not shown). In contrast, MGA271 did not mediate ADCC against the B7-H3–negative Raji B-cell lymphoma.

**MGA271 exhibits potent in vivo efficacy against renal cell carcinoma xenografts**

To evaluate the antitumor activity of MGA271 in vivo, xenograft studies were carried out in mice that have the murine CD16 gene knocked out and are transgenic for human CD16A-158F, the low-affinity binding allele of this FcR (mCD16^{−/−} hCD16A^−). This model was developed to overcome the discrepancy in binding of the MGFc0264 Fc domain to murine CD16, compared with the human CD16A counterpart (13), and provides the greatest sensitivity for evaluating the potential enhanced Fc-dependent activity mediated by mAbs with engineered Fc domains. The pharmacokinetics of MGA271 was evaluated at a dose of 5 mg/kg in mCD16^{−/−} hCD16A FOXN1 nu/nu mice. The estimated half-life of MGA271 was 249 hours with a C_{max} of 43 µg/mL, supporting once weekly administration of MGA271 in xenograft efficacy models.

Treatment with MGA271 once weekly for 5 weeks at doses ranging from 0.1 to 10 mg/kg, initiated 7 days following tumor cell implantation, resulted in sustained inhibition of the growth of A498 tumor xenografts over the course of the 50-day study (Fig. 5A). A cytostatic response was achieved over the course of the study at doses of 0.5 mg/kg or greater, with a near cytostatic response achieved at the 0.1 mg/kg dose.

Efficacy studies were also carried out against 786-0 renal cell carcinoma and HT-1197 bladder carcinoma tumor xenografts. Treatment with MGA271 once weekly for 5 weeks at doses of 1, 2.5, and 5 mg/kg initiated 7 days following tumor cell implantation resulted in sustained inhibition of the growth of 786-0 tumor xenografts over the course of the study (Fig. 5B). Tumor growth inhibition was also observed with RES240 in this study; however, growth inhibition by MGA271 was significantly greater than that observed with the RES240, indicating that the MGFc0264 Fc mediated enhanced antitumor activity in vivo. The tumor growth inhibition observed was Fc mediated, as treatment with RES240-aglycosyl did not inhibit tumor growth (Fig. 5C). Potent antitumor activity was also observed against HT-1197 tumor xenografts. Treatment with MGA271 once weekly for 5 weeks at doses of 1, 5, and 10 mg/kg, initiated 7 days following tumor implantation, led to sustained inhibition of the growth of HT-1197 tumor xenografts over the course of the study (Fig. 5D). The antitumor activity of MGA271 was also evaluated in a latetreatment mode, wherein the 786-0 tumor xenografts were allowed to grow to an average volume of approximately 260 mm^3 before treatment. As shown in Fig. 5E, once weekly treatment with MGA271 at doses of 1 mg/kg or greater resulted in a significant inhibition of tumor growth. At 5 or 10 mg/kg, a cytostatic response was achieved until day 52, after which the average tumor volume of the 5 mg/kg treatment group remained near predose administration levels, whereas the 10 mg/kg group exhibited a nonsignificant trend toward relapse.

**Toxicokinetic and toxicology assessment in cynomolgus monkeys**

The toxicokinetic and toxicologic profile of MGA271 was assessed in cynomolgus monkeys following a single
administration and 4 once weekly administrations of MGA271. Cynomolgus monkeys exhibit MGA271 reactivity comparable with that observed with human tissues. In addition, cynomolgus monkey FcRs bind to the optimized Fc domain of MGA271 (13), and as previously shown (Fig. 3D), cynomolgus monkey PBMCs were capable of mediating MGA271-dependent ADCC of a magnitude similar to that mediated by human PBMCs. No significant adverse MGA271-mediated changes were observed following a single administration and 4 once weekly administrations of MGA271 at dose levels up to 150 mg/kg, the highest dose examined. Minor transient MGA271-related elevations in the serum level of interleukin 5 (IL-5), IL-6, and TNF-α were observed following administration of MGA271 (Supplementary Fig. S3), which were not accompanied by clinical symptoms. A time- and dose-dependent recoverable reduction in circulating natural killer cells was observed following administration of MGA271 (Supplementary Fig. S4) that, owing to the transient nature of the response, was not considered adverse. On the basis of these results, the no-observed-adverse-effect level (NOAEL) in cynomolgus monkeys for MGA271 was established at 150 mg/kg. The mean terminal half-life ranged from 8 to 12 days.

Discussion

We previously outlined an approach to generate mAbs directed against cell-surface antigens overexpressed in cancer, based on whole-cell immunization and selection for favorable tumor:normal tissue expression (8). These mAbs recognize a spectrum of potential targets with some linked to a functional role in tumor formation and/or progression (e.g., adhesion molecules, growth factor receptors, receptor tyrosine kinases, metabolic targets, and metalloproteases) and others with an undiscovered role in tumorigenesis. The selection of highly tumor-specific targets enables the latter

Figure 4. MGA271 mediates in vitro ADCC across a panel of ATCC cancer cell lines exhibiting a range of B7-H3 expression. The ability of MGA271 to mediate ADCC was evaluated on B7-H3–positive cancer cell lines with resting PBMCs. A, LnCAP prostate adenocarcinoma; B, SK-MES-1 lung carcinoma; C, MDA-MB-468 breast adenocarcinoma; D, SW780 colon adenocarcinoma; E, ACHN renal cell carcinoma; F, HT-1197 bladder carcinoma; G, UACC-62 melanoma; H, 786-0 renal cell carcinoma; I, B7-H3–negative Raji B cell lymphoma.
category of antigens to be candidates for Fc-optimization to enhance the ability of the mAb to bind to activating Fcγ receptors and mediate enhanced Fc-dependent antitumor activities.

In this article, we have described identification and characterization of a panel of mAbs with specificity for B7-H3, a protein overexpressed on many cancers, including prostate (15–17), renal (18), pancreatic (19, 20), colorectal (21), non–small cell lung (NSCLC; ref. 22), ovarian (23), bladder (24), melanoma (25), and neuroectodermal (25) cancers and postulated to mediate immunomodulatory activity (26). Our immunohistochemical analysis confirmed and extended published data indicating B7-H3 is highly expressed across a variety of solid cancers. We observed high levels of B7-H3 expression in kidney, prostate, pancreatic, breast, gastric, and ovarian cancer, as well as melanoma, but limited expression in normal human tissues. B7-H3 expression was observed in the epithelial compartment of the tumor as well as the tumor vasculature. It is worth noting that genetic expression screens would not have identified B7-H3 as a tumor-specific target. B7-H3 mRNA has been reported to be broadly expressed across normal organ and immune tissues (27), whereas B7-H3 protein expression is more limited in normal tissues. Consistent with our study, 2 independent reports describe differential B7-H3 protein expression on tumor tissues compared with normal tissues. A membrane-bound tumor-associated antigen defined by the mAb 376 (28) and subsequently identified as B7-H3 (29) was reported to be expressed on melanoma, glioma, neuroblastoma, sarcoma, and breast cancer cells and tissues, but minimally or undetectably expressed on adult normal tissues (28, 30). Modak and colleagues (25) reported that the 8H9 mAb, whose binding antigen was ultimately determined to be B7-H3 (31), exhibited undetectable binding in most normal tissues and only limited cytoplasmic staining in normal pancreas, stomach, liver, and adrenal cortex.
Evidence suggests that posttranslational regulation by the miRNA miR-29 may contribute to the observed lack of correspondence between B7-H3 mRNA and protein expression levels (31).

The functional role of B7-H3 remains largely undissected and controversial. Reports suggesting a costimulatory role in immune modulation for B7-H3 (27) have been countered by a preponderance of evidence suggesting a tumor-supportive role in terms of immunologic escape (11, 32–34) and increased resistance to treatment (35). Overexpression of B7-H3 has been correlated with disease severity and poor outcome in a growing number of cancer types, including pathologic indicators of aggressiveness and negative clinical outcome in prostate cancer (15–17), increased grade and decreased T-lymphocyte infiltration in tumor nests and stroma in colorectal cancer (21), and a reduction in T-lymphocyte infiltrates in NSCLC (22). B7-H3 expression on tumor-associated vascular endothelia suggests additional roles for B7-H3 that favor tumor growth and progression, albeit by mechanism(s) not yet uncovered.

Consistent with this notion, the presence of B7-H3 on the epithelial or vasculature component of renal cell carcinoma was associated with multiple adverse clinical and pathologic features and an increased risk of death (18). In addition, in ovarian cancer, B7-H3 expression in tumor vasculature was associated with significantly shorter patient survival and higher incidence of disease recurrence (23). Tumor-specific effects of B7-H3 have also been described. Two studies have shown that siRNA knockdown of B7-H3 expression reduced cell adhesion, migration, and invasion of melanoma and breast cancer cells (29) and prostate cancer cells (36) in vitro, suggesting B7-H3 may play a role in tumor progression and metastasis. Silencing of B7-H3 expression has also been reported to regulate intracellular signaling of breast cancer cells and modulate chemotherapy resistance (34). The derivation of a B7-H3 mAb from an ovarian cancer line displaying cancer stem cell (CSC) properties (Table 1; ref. 37) also warrants exploration of the expression and potential biologic role for B7-H3 on CSCs and whether B7-H3 can serve as a therapeutic target for CSCs in addition to the broader tumor cell population.

Because the role of B7-H3 in tumorigenesis and immune escape is not fully understood, and given the exquisite tumor-specific expression of the target, the clinical candidate mAb BRCA84D was engineered with an Fc region that imparts increased affinity for the human activating Fcγ receptor CD16A and decreased affinity for the human inhibitory Fcγ receptor CD32B (12, 13). This choice reflects the notion that enhancing the ability of an mAb to mediate Fc-dependent activity may translate to improved clinical efficacy—a concept supported by clinical data that correlate clinical outcome with Fcγ receptor polymorphisms. Specifically, patients homozygous for the higher affinity variant of CD16A or CD32A have more favorable clinical outcomes following treatment with rituximab for follicular lymphoma (4, 5), trastuzumab for metastatic breast cancer (6), or cetuximab for mCRC under certain settings (7). In the latter case, KRAS mutations were associated with lower response rates to cetuximab; however, the high-affinity polymorphism was associated with more positive outcomes irrespective of KRAS status. Thus, cetuximab mediates a positive clinical response in mutated KRAS mCRC under conditions in which the EGFR pathway is constitutively active and insensitive to the direct cytostatic mechanism of the antibody, supporting the importance of the role of Fc-mediated mechanisms in the efficacy of cetuximab.

Consistent with the enhanced binding of MGA271 to CD16A, MGA271 exhibited enhanced ADCC activity against all B7-H3–positive cancer cell lines evaluated when compared with forms of the mAb with a wild-type Fc domain. Although strong correlations between B7-H3 expression and sensitivity to MGA271-mediated ADCC cannot be made based on the tumor cell lines tested in this study, overall the cell lines expressing the greatest level of cell-surface B7-H3 exhibited the greatest sensitivity. The cytotoxic activity observed in vitro was reflected in the potent antitumor activity in xenograft models in vivo. Significant inhibition of tumor growth and cytostasis with xenografts representing renal cell carcinoma and bladder carcinoma was observed following weekly administration of MGA271. The antitumor activity was dependent on the Fc region of the mAb and was enhanced in comparison with a wild-type Fc version. Significant antitumor activity was also observed against a variety of additional xenografts representing tumors of gastric, lung and colon cancer, and melanoma (data not shown). Consistent with the limited expression of B7-H3 in normal tissues, MGA271 was well tolerated in cynomolgus monkeys, and the NOAEL was determined to be 150 mg/kg.

The focus of our approach to developing MGA271 as an anti-B7-H3 mAb therapy has been on exploiting the favorable tumor/normal expression profile of B7-H3 to provide a therapeutic window for enhanced tumor cell killing through Fc-engineering. However, it is important to note that MGA271 retains the potential to target additional mechanisms of action, including directly modulating tumor cell functions, mediating antitumor vasculature activities, and modulating immune suppressive activity. These areas of potential therapeutic intervention remain to be explored and will be greatly facilitated by identification of the B7-H3 receptor.

In summary, we have developed MGA271, a B7-H3-reactive, Fc-engineered mAb that mediates potent antitumor activity in vitro as well as in tumor xenograft studies; these data, together with its favorable safety profile in cynomolgus monkey toxicity studies, support the exploration of MGA271 in the treatment of B7-H3–positive cancers. A phase I/IIa clinical study of MGA271 in patients with B7-H3–positive metastatic or recurrent adenocarcinoma has been initiated.

Disclosure of Potential Conflicts of Interest

Koenig has held the title of president and CEO of MacroGenics Inc. The other authors disclosed no potential conflicts of interest.

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References


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