Engineered anti-CD70 antibody-drug conjugate with increased therapeutic index

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Abstract
An anti-CD70 antibody conjugated to monomethylauristatin F (MMAF) via a valine-citrulline dipeptide containing linker has been shown previously to have potent antitumor activity in renal cell cancer xenograft studies. Here, we generated a panel of humanized anti-CD70 antibody IgG variants and conjugated them to MMAF to study the effect of isotype (IgG1, IgG2, and IgG4) and Fcγ receptor binding on antibody-drug conjugate properties. All IgG variants bound CD70+ 786-O cells with an apparent affinity of ~1 nmol/L, and drug conjugation did not impair antigen binding. The parent anti-CD70 IgG1 bound to human FcγRI and FcγRIIa V158 and mouse FcγRIV and this binding was not impaired by drug conjugation. In contrast, binding to these Fcγ receptors was greatly reduced or abolished in the variant, IgG1v1, containing the previously described mutations, E233P:L234V:L235A. All conjugates had potent cytotoxic activity against six different antigen-positive cancer cell lines in vitro with IC50 values of 30 to 540 pmol/L. The IgG1v1 conjugate with MMAF displayed improved antitumor activity compared with other conjugates in 786-O and UMRC3 models of renal cell cancer and in the DBTRG05-MG glioblastoma model. All conjugates were tolerated to ≥40 mg/kg in mice. Thus, the IgG1v1 MMAF conjugate has an increased therapeutic index compared with the parent IgG1 conjugate. The improved antitumor activity of the IgG1v1 auristatin conjugates may relate to increased exposure as suggested by pharmacokinetic analysis. The strategy used here for enhancing the therapeutic index of antibody-drug conjugates is independent of the antigen-binding variable domains and potentially applicable to other antibodies.

Introduction
The therapeutic concept of antibody-drug conjugates (ADC) is to use an antibody to deliver a cytotoxic payload to tumor cells via binding to a target cell surface antigen (1, 2). ADC internalization and release of the cytotoxic drug is commonly required to kill the target cell. ADCs show significant potential for enhancing the antitumor activity of “naked” antibodies and for reducing the systemic toxicity of drugs as shown by numerous preclinical examples (3). Moreover, clinical demonstration of the ADC concept is provided by gemtuzumab ozogamicin (Mylotarg), a humanized anti-CD33 antibody conjugated to calicheamicin, approved for the treatment of acute myeloid leukemia (4). At least 10 ADCs are in clinical trials, including Mylotarg (3).

Potent cytotoxic drugs incorporated into ADCs, including calicheamicin used in gemtuzumab ozogamicin, which induces double-stranded DNA cleavage, as well as auristatins and maytansinoids, which inhibit tubulin polymerization (1, 2). The most commonly used isotype for the antibody delivery vehicle is IgG1. However, ADCs constructed with antibodies of all human IgG isotypes, except IgG3, are currently in clinical trials (3). No systematic preclinical comparison of ADCs of different isotypes has been reported previously.

Human IgG of different isotypes differ in their ability to support secondary immune functions, antibody-dependant cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity. IgG1 can potentially support ADCC and complement-dependent cytotoxicity, whereas IgG2 and IgG4 are typically inefficient in their effector functions (5). For ADCs, effector functions could potentially be advantageous by providing additional antitumor activities or conversely disadvantageous, if effector cell binding reduces ADC localization to tumors, impairs ADC internalization by tumor cells, or gives rise to additional toxicities.

The humanized anti-CD70 IgG1 antibody, h1F6 (SGN-70), has potent in vitro ADCC and complement-dependent cytotoxicity activities (36). Anti-CD70 ADCs containing monomethylauristatin F (MMAF) and other auristatins have potent in vitro antitumor activity in xenograft studies of renal cell cancer (6). Here, IgG2 and IgG4 variants of h1F6 were generated to investigate the effect of IgG isotype on ADC activities. Additionally, IgG1 and IgG4 variants...
were mutated in their Fc regions to generate IgG1v1 and IgG4v3, respectively, to study the influence of Fcγ receptor binding on ADC properties. The anti-CD70 IgG variants were conjugated to MMAF (7) and compared in vitro with respect to their antigen-binding affinity, human and murine Fcγ receptor (huFcγR and muFcγR) binding, and cytotoxic activity against cancer cell lines. Subsequently, the ADCs were compared in vivo in pharmacokinetic, efficacy, and safety studies.

Materials and Methods

Cell Lines

Cancer cell lines were obtained from the following sources: UMRC3 (Dr. John A. Copland, Mayo Clinic College of Medicine); DBTRG05-MG (European Collection of Cell Cultures); THP-1, 786-O, Caki-1, and HCT 116 (American Type Culture Collection); and L-428 and LP-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen). THP-1, 786-O, L-428, LP-1, DBTRG05-MG, and HCT 116 cell lines were cultured at 37°C with 5% CO2 in RPMI 1640 supplemented with 10% fetal bovine serum. Caki-1 cells were cultured at 37°C with 5% CO2 in McCoy’s 5a supplemented with 10% fetal bovine serum. UMRC3 cells were cultured at 37°C with 5% CO2 in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate.

Construction and Expression of Anti-CD70 Antibody Variants and Fcγ Receptors

The generation of h1F6 (SGN-70), a humanized IgG1-anti-CD70 antibody, has been described (36). Isotype switch variants were created by PCR by fusing the h1F6 VH gene to sequences encoding human IgG2 or IgG4 (8). The anti-CD70 IgG1v1 variant containing the mutations S228P:L235A:G237A:E318A (10) were generated using the Pymol mutagenesis wizard to provide space for hinge region of 1HZH were built into the structure and the T236, H237, and T238) missing from one heavy-chain (RIIIB, was generated using

Antibody Purification

The anti-CD70 IgG variants were purified by protein A chromatography using an AKTAexplorer FPLC (GE Healthcare) as described (13) with modifications provided in Supplementary Materials.1 Purified antibodies were analyzed by SDS-PAGE and TSK-Gel G3000SW high-performance liquid chromatography size exclusion chromatography (Tosoh Bioscience).

Antibody Conjugation

ADCs were prepared following partial reduction of antibody interchain disulfide bonds with Tris(2-carboxyethyl)-phosphine (TCEP) as described (14) with the following modifications. Antibody preparations in PBS were concentrated to 10 mg/mL. Partial reduction of the interchain disulfide bonds, to a mean of 2 reduced disulfide bonds or 4 reactive thiols per antibody, was achieved by incubating antibody solutions with TCEP at 37°C in the presence of 1 mmol/L diethylentriaminepentaacetic acid. The molar ratio of TCEP to antibody and the incubation time necessary to achieve the desired degree of reduction was optimized for the different IgG isotypes. The IgG reduction state was determined by conjugating a small aliquot followed by hydrophobic interaction chromatography (15). Incubation of IgG1 and IgG1v1 variants with 2.5 TCEP equivalents for 1.5 h yielded 4.0 ± 0.4 free thiols per antibody. IgG4 and IgG4v3 variants required 4 h incubations with 4 TCEP equivalents and the IgG2 variant needed an incubation of 5 h with 6 equivalents of TCEP to achieve similar yields of free thiols.

Reduced antibodies were conjugated for 30 min on ice with a 1.5-fold molar excess of either maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl-MMAF (mcvcMMAF) or maleimidocaproyl-MMAF (7) from DMSO stock solutions. The conjugation reaction was quenched with a 5-fold molar excess of N-acetylcysteine and the resulting conjugates purified by ion exchange using VivaPure 5 cation exchange membrane centrifugal devices (Sartorius). Conjugates were diluted 10-fold with 20 mmol/L sodium acetate (pH 5.0) and bound to the membrane, washed with additional sodium acetate buffer, and eluted with 3× PBS. Drug loading was determined by reverse-phase high-performance liquid chromatography under reducing conditions (13, 14) and by hydrophobic interaction chromatography (15).

To prepare fluorescently labeled conjugates, antibodies were reduced as described above and a 1.5-molar excess of Alexa Fluor 488 maleimide (Invitrogen) added from a 2 mmol/L stock solution in DMSO. After 30 min on ice, the reaction was quenched with a 5-fold molar excess of N-acetylcysteine. Conjugates were purified by three rounds of dilution with PBS and centrifugal ultrafiltration (Amicon Ultra, 30-kDa MWCO), with each round achieving ≥10-fold initial dilution of the antibody solution.

Molecular Modeling

A molecular model of an IgG1-vcF8 ADC complexed with the human receptor, FcγRIIIB, was generated using Pymol (16). The ADC model, comprised a human IgG1 conjugated to 8 equivalents of mcvcMMAF (7), was generated using the atomic coordinates of the human IgG1, B12 (PDB accession code: 1HZH; ref. 17). The three residues (T236, H237, and T238) missing from one heavy-chain hinge region of 1HZH were built into the structure and the resulting backbone geometry regularized using Coot (18). The four interchain disulfide bonds were broken in silico and alternate backbone-dependent rotamers sampled using the Pymol mutagenesis wizard to provide space for

1 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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modeling drug. Atomic coordinates for mc-vcMMAF were generated and energy minimized using Chem3D (CambridgeSoft). Next, mc-vcMMAF was connected to each of the eight target cysteine residues: C220, C226, and C229 in the two heavy chains and C214 in the two light chains (Kabat light chain and EU heavy chain numbering; ref. 19). This was accomplished after positioning one of the Michael acceptor maleimide carbon atoms from mc-vcMMAF 1.8 Å away from each of the eight cysteinyl sulfurs.

The receptor, huFcRIIIb, was docked with the IgG1-vcF8 ADC model. Appropriate positioning of the receptor was determined by superimposing the Fc components of the Fc/huFcRIIIb complex (PDB accession code: 1E4K; ref. 20) on corresponding parts of the ADC model. Two possible orientations result from this superposition due to the internal 2-fold symmetry within the Fc. The root mean square deviation was calculated for each (2.28 and 1.17 Å) and the closer fitting superposition used for further modeling. Coordinates for huFcRIIIb were extracted from the favored superposition and viewed in the context of the ADC. The huFcRIIIb was moved (<10 Å on any axis) away from the ADC to relieve steric clashes between the two models.

**In vitro Characterization of Anti-CD70 IgG Variants and Their Drug Conjugates**

Binding of fluorescently labeled anti-CD70 IgG variants to antigen-positive 786-O cells was undertaken to assess the effect of Fc mutation and isotype switching on antigen binding affinity. Each IgG variant was reduced and labeled with Alexa Fluor 488 C6 maleimide (see above). 786-O cells were then combined with serial dilutions of each fluorescently labeled IgG variant in PBS, 0.1% bovine serum albumin (w/v) buffer and incubated for 30 min on ice in the dark. Cells were washed with PBS, 0.1% bovine serum albumin (w/v) buffer and labeled cells detected using a LSRII fluorescence-activated cell sorting analyzer (Becton Dickinson Biosciences). Saturation binding data were fitted to a one site binding model using Prism v4.01 (GraphPad Software) to determine the apparent binding affinities.

The effect of drug conjugation on antigen binding by IgG variants was evaluated by modifying this cell binding assay to a competition format. 786-O cells were combined with serial dilutions of the anti-CD70 IgG1 parent antibody, IgG variants, or corresponding drug conjugates in the presence of 225 ng/mL anti-CD70 IgG1 labeled with Alexa Fluor 488. Flow cytometry data, collected as described above, are reported as the percentage of maximum fluorescence calculated by the sample fluorescence divided by the fluorescence of cells stained with 225 ng/mL anti-CD70 IgG1-Alexa Fluor 488 alone.

Cell-based competition binding assays were established to study the interactions of anti-CD70 IgG variants and their drug conjugates with huFcRI, the high-affinity form of huFcRIIa V158 and muFcRIV. Stable CHO DG-44 cell lines expressing huFcRI, huFcRIIaV158 or muFcRIV were combined with 10, 50 or 200 nmol/L Alexa Fluor 488 labeled anti-CD70 IgG1, respectively, in the presence of serial dilutions of each of the IgG variants and their drug conjugates in PBS, 0.1% bovine serum albumin (w/v) buffer and incubated for 30 min on ice in the dark. Labeled cells were detected using a LSRII fluorescence-activated cell sorting analyzer and IC50 values estimated by a nonlinear least squares fit of the data to a four-variable logistic equation using Prism v4.01.

Growth inhibition of CD70+ 786-O, Caki-1, L-428, UMRC-3, LP-1, and DBTRG05-MG cells and CD70+ HCT 116 cells was determined by incubating cells with ADCs for 92 h followed by addition of 50 μmol/L resazurin for 4 h at 37°C. Dye reduction was measured using a Fusion HT microplate reader (Packard Instruments). IC50 values were estimated as described above. The CD70 copy number per cell was estimated by quantitative flow cytometry using a previously described method (21).

**Pharmacokinetics**

ADC pharmacokinetic properties were evaluated in nude (nu/nu) mice. Groups (n = 3) of nude mice (Harlan) were administered with 10 mg/kg ADCs based on the antibody component by tail vein injection. Blood samples were collected from each mouse via the saphenous vein at 0.5 and 4 h and at 1, 2, 3, 7, 14, 21, 28, 34, 49, and 63 days after injection and serum was isolated. All animal experiments were conducted under Institutional Animal Care and Use Committee guidelines and approval. Serum concentrations of ADCs were measured by sandwich ELISA for total antibody using an anti-1F6 (anti-idiotype) antibody coat, followed by biotinylated anti-1F6, and horseradish peroxidase-conjugated streptavidin for detection (see Supplementary Materials).1 Serum concentration data for each animal were analyzed using WinNonlin version 4.0.1 (Pharsight).

**Efficacy Models**

Nude (nu/nu) female mice (Harlan) were implanted s.c. with 1 × 10⁶ 786-O, DBTRG05-MG, or UMRC3 cells. When the donor tumors were ~500 mm³, mice were euthanized and the tumors were aseptically excised. Tumors were sectioned into ~0.5 × 0.5 mm fragments, loaded into a sterilized 13-gauge trocar, and injected into the right flank of recipient mice. Tumors were serially passaged for 11 generations and used for study implant from generations 2 to 11. For efficacy studies, animals were implanted with tumor fragments as described above. When the mean tumor volume was ~90 mm³, tumor-bearing mice were randomly divided into groups of eight to nine animals and treatment was initiated by i.v. administration. For the 786-O model, mice were dosed once with an ADC at 0.50 or 1.5 mg/kg for vcF4 conjugates or alternatively at 1.5 or 4.5 mg/kg for mcF4 conjugates. For the DBTRG05-MG model, mice were dosed once with vcF4 conjugates at 3.0 or 10 mg/kg. For the UMRC3 model, mice were dosed every 4 days times 4 with a vcF4 conjugate at 3.0 or 6.0 mg/kg or alternatively left untreated. Control groups included no treatment, nonbinding control conjugates (vcF4 or mcF4), and nonconjugated anti-CD70 IgG1. Tumor size was measured twice weekly by using calipers. Tumor volume was calculated using the formula: \(V = \frac{A \times B^2}{2}\), where \(A\) and \(B\) are the largest and second largest perpendicular tumor
dimensions, respectively. Animals were euthanized when tumors reached ≥1,000 mm³ or at the end of the study.

**Maximum Tolerated Dose**

Groups of BALB/c mice (n = 3) were injected with 40, 60, or 80 mg/kg ADCs via the tail vein to determine the single dose maximum tolerated dose. Mice were monitored daily for 14 days, and both weight and clinical observations were recorded. Mice that developed significant signs of distress were euthanized.

**Statistical Analysis**

The log-rank (Mantel-Cox) test was applied using Prism 5.0 (GraphPad Software) to analyze the differences in median tumor quadrupling time between groups. Differences were deemed significant when P < 0.05. Tumor quadrupling times were determined by nonlinear regression analysis for exponential growth for each experimental animal. Animals that did not reach the quadrupling endpoint were assigned a quadrupling time as the last day of the study.

**Results**

**Generation of Anti-CD70 IgG Variants**

The parent anti-CD70 antibody for this study, h1F6 (SGN-70), is a humanized IgG1 with potent ADCC and complement-dependent cytotoxicity activities (36). IgG2 and IgG4 variants of h1F6 were generated to investigate the effect of antibody isotype on ADC activities. Additional IgG variants were made to study the influence of interactions with Fcγ receptors on ADC properties. Specifically, an IgG1 variant (IgGlv1) was generated that contains the mutations, E233P:L234V:L235A, which greatly impair binding to huFcγRI, huFcγRIIA, huFcγRIIB, and huFcγRIIA with only modestly impaired (~2-fold) binding to the human salvage receptor, FcRn (9, 22). Four mutations, S228P:L235A:G237A:E318A (10), were introduced into IgG4 variant to generate IgG4v3. The three alanine substitutions greatly impair binding to huFcγR and the complement component, C1q (10), whereas the S228P mutation promotes interheavy over intraheavy chain disulfide bonding (refs. 23, 24; see below).

All antibody variants were stably expressed in CHO-DG4 cell lines at titers of 15 to 35 mg/L. The antibody variants were purified from 0.5 to 10 L cultures by protein A chromatography in yields of 70% to 95% with >95% purity as assessed by SDS-PAGE (Supplementary Fig. S1).1 All anti-CD70 antibody variants electrophoresed under reducing conditions gave two major bands consistent with the presence of heavy and light chains. Under nonreducing conditions, all IgG variants electrophoresed as a single major band, as anticipated. The IgG4 variant gave rise to an additional minor band, consistent with the presence of “half-IgG,” as reported for other recombinant IgG4 and attributed to inefficient interheavy chain disulfide bond formation (23, 24). Mutation of the IgG4 hinge sequence, CPSC, to mimic the IgG1 hinge, CPPC, creates a strong preference for interheavy over intraheavy chain disulfide bonding (23–25). IgG4v3 includes the S228P mutation and migrates as a single major band under nonreducing conditions, as anticipated (23–25). All IgG variants bound to CD70⁺ 786-O cells, with a ~1 nmol/L apparent affinity (Table 1). Thus, the IgG isotype, hinge, and Fc region point mutations did not alter the antigen binding affinity, as anticipated, because the antigen-binding variable domains are identical in all IgG variants.

**Preparation of ADCs**

IgG variants were partially reduced with TCEP to yield a mean of 4 free thiol groups/antibody and then reacted with mc-vcMMAF (7). The reduction conditions were optimized for each IgG isotype: 1.5 h with 2.5 equivalents of TCEP for IgG1 and IgG1v1 and 4 h with 4.0 equivalents of TCEP for IgG4 and IgG4v3, whereas the IgG2 required 5 h with 6.0 equivalents of TCEP. These varied reduction requirements likely reflect amino acid sequence differences such as those in the hinge region and altered disulfide bonding patterns of IgG variants. IgG2 may form four interheavy chain disulfide bonds, whereas only two are possible for IgG1 and IgG4. The mean drug loading for each conjugate was close to the expected 4 drugs/antibody (Table 1). The anti-CD70 ADCs with mc-vcMMAF will be referred to hereafter by their IgG variant name with the vcF4 suffix (e.g., IgG1-vcF4 represents the ADC of the parent humanized IgG1 antibody, h1F6, conjugated to 4 equivalents of mc-vcMMAF). The ADCs contained only low levels of aggregates (≤2.5%) as assessed by analytical size-exclusion chromatography (Table 1). Competition binding experiments with 786-O cells and fluorescently labeled anti-CD70 IgG1 revealed that drug conjugation did not impair binding by any of the IgG variants (Table 1), as reported for other ADCs (13). This presumably reflects that the solvent accessible cysteine residues used for drug conjugation are distant from the antigen-binding residues in the variable domains.

**Binding of IgG Variants and Their Drug Conjugates to Fcγ Receptors**

Interaction of the IgG variants and their corresponding drug conjugates with muFcγR and huFcγR was investigated with a view to tumor xenograft studies and potential future clinical applications of ADCs, respectively. Binding of fluorescently labeled anti-CD70 IgG1 to muFcγR and huFcγR expressed recombinantly on the surface of CHO cells was competed with the IgG variants and their drug conjugates. Competition binding assays were developed for muFcγRIIV, huFcγRII, and the high-affinity form of huFcγRIIA V158, whereas binding of the fluorescently labeled IgG1 variant to muFcγRI, muFcγRIIB, and huFcγRIIA was too weak to establish robust assays (data not shown).

The IgG1 parent antibody competed efficiently for binding to the murine receptor, muFcγRIIV, and this binding was not impaired by drug conjugation (Fig. 1A). Similarly, no competition was observed for any of the IgG variants or their corresponding ADCs with muFcγRIIV (Fig. 1A). However, several of the nonconjugated antibodies showed a modest augmentation of the fluorescent signal at the highest concentrations tested for reasons yet unknown.
As for the human receptors, the IgG1 parent competed efficiently for binding to huFcyRI and huFcyRIIIA V158, and this binding was not impaired by drug conjugation (Fig. 1B and C). This observation was anticipated from the physical separation of all eight potential drug conjugation sites (solvent accessible cysteines) on an IgG1 and the Fc receptor binding sites in a molecular model of a human IgG1-vcF8 ADC bound to huFcyRIIIIB (Fig. 1D), generated from X-ray crystallographic structures of a human IgG1 Fc region in complex with huFcyRIIIIB (20), and B12, a human IgG1 (17).

As expected for IgG1v1 (9, 22), reduced or minimal binding competition was observed with huFcyRIIIA V158 and huFcyRI, respectively, with similar findings for IgG1v1-vcF4, the corresponding conjugate (Fig. 1B and C). IgG4 and its conjugate, IgG4-vcF4, displayed comparable and efficient competition binding to huFcyRI that was abolished in IgG4v3 and its corresponding drug conjugate, IgG4v3-vcF4 (Fig. 1B). Negligible competition binding was observed for IgG4, IgG4v3, and their corresponding drug conjugates with huFcyRIIIA V158 (Fig. 1C). IgG2 and IgG2-vcF4 showed minimal competition binding to either huFcyRI or huFcyRIIIA V158 (Fig. 1B and C).

**ADC In vitro Cytotoxic Activity**

All anti-CD70 ADCs had potent in vitro cytotoxic activity (30-540 pmol/L IC50 values) against six different cancer cell lines expressing 34,000 to 189,000 copies of CD70 per cell (Fig. 2). This cytotoxic activity reflects drug conjugation because none of the nonconjugated IgG variants had detectable cytotoxic activity against any of these cell lines. The ADC-sensitive cancer cell lines included 786-O (renal carcinoma), was not sensitive (IC50 >> 7 nmol/L) to any anti-CD70 ADCs but was sensitive (IC50 = 2 nmol/L) to a positive control ADC (OKT9, anti-transferrin receptor; data not shown).

**Pharmacokinetics of ADCs**

Immunodeficient (nude) mice were dosed with anti-CD70 IgG variant ADCs to probe the effect of isotype and point mutations on pharmacokinetics. Immunodeficient mice were chosen to circumvent an antibody response to the ADCs and for consistency with subsequent efficacy experiments. Nude mice lack endogenous IgG, consequently administered IgG may potentially bind to endogenous FcyR on a variety of tissues and normal cells. Although this possibility was not explored here, it seems unlikely to have a major influence, because similar pharmacokinetic properties have been observed for chimeric antibodies in both immunocompetent (BALB/c) and immunodeficient (SCID) mice (26).

Pharmacokinetic data (Fig. 3) were subjected to non-compartmental analysis to estimate the terminal half-life, area under the curve (AUC), and clearance (Table 2). IgG1-vcF4, IgGv1-vcF4, and IgG2-vcF4 had similar terminal half-lives (5-6 days) that were longer than for IgG4-vcF4 and IgG4v3-vcF4 (3-4 days). The AUC was largest and similar for IgG1v1-vcF4 and IgG2-vcF4 and about 2-, 2.5-, and 4.5-fold lower for IgG1-vcF4, IgG4-vcF4, and IgG4v3-vcF4, respectively. Compartmental analysis revealed a biphasic disposition for all IgG variant ADCs with a transition from α to β phases at ~3 days after dosing. The AUC from 0 to 3 and to infinity days were used to estimate the α- and β-phase contributions to AUC, respectively. The β phase contributed 64% and 73% of the AUC for IgG1v1-vcF4 and IgG2-vcF4, respectively, and only 36% to 40% of the AUC for IgG1-vcF4, IgG4-vcF4, and IgG4v3-vcF4 (Table 2). Clearance (dose/AUC) was slowest for IgG1v1-vcF4 and IgG2-vcF4 and increased about 2-, 2.5-, and 4.5-fold for IgG1-vcF4, IgG4-vcF4, and IgG4v3-vcF4, respectively.

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**Table 1. In vitro properties of anti-CD70 IgG variants and IgG-vcF4 drug conjugates**

<table>
<thead>
<tr>
<th>h1F6 IgG variant</th>
<th>KD IgG (nmol/L)</th>
<th>IC50 ADC/IC50 IgG</th>
<th>Drugs/antibody method 1, method 2</th>
<th>ADC monomer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>1.20 ± 0.22</td>
<td>1.03 ± 0.13</td>
<td>3.9 ± 0.0, 4.2 ± 0.1</td>
<td>99.5 ± 0.3</td>
</tr>
<tr>
<td>IgG1v1</td>
<td>1.19 ± 0.24</td>
<td>1.05 ± 0.03</td>
<td>3.7 ± 0.1, 4.1 ± 0.1</td>
<td>98.9 ± 0.4</td>
</tr>
<tr>
<td>IgG2</td>
<td>0.87 ± 0.20</td>
<td>0.96 ± 0.06</td>
<td>3.9 ± 0.1, 4.0 ± 0.3</td>
<td>97.5 ± 1.2</td>
</tr>
<tr>
<td>IgG4</td>
<td>1.07 ± 0.20</td>
<td>0.88 ± 0.02</td>
<td>4.0 ± 0.1, 4.3 ± 0.2</td>
<td>97.6 ± 0.6</td>
</tr>
<tr>
<td>IgG4v3</td>
<td>1.17 ± 0.16</td>
<td>1.23 ± 0.10</td>
<td>4.3 ± 0.3, 4.6 ± 0.4</td>
<td>98.1 ± 0.6</td>
</tr>
</tbody>
</table>

NOTE: Mean ± SE from three or more independent experiments with the anti-CD70 IgG variants and their corresponding conjugates with the linker-auristatin drug, mc-vcMMAF (7).

aVariants of the humanized anti-CD70 antibody, h1F6, are designated by their IgG isotype. The IgG1v1 and IgG4v3 variants contain the mutations, E233P:L234V:L235A (9, 22) and S228P:L235A:G237A:E318A (10), respectively, using the EU numbering scheme of Kabat (19).

bAnti-CD70 IgG variants conjugated to Alexa Fluor 488 were incubated at varying concentrations with 786-O cells. KD values were estimated from saturation binding data using a one site binding model (Prism v4.01).

bRatio of IC50 values for competition binding with anti-CD70 IgG1-Alexa Fluor 488 binding to 786-O cells.

bADC loading with auristatin was determined by reverse-phase high-performance liquid chromatography (method 1; refs. 13, 14) and hydrophobic interaction chromatography (method 2; ref. 15).

bADC percent monomer was estimated from the peak areas in size exclusion chromatography.
ADCC Efficacy in Tumor Xenograft Models of Renal Cell Cancer and Glioblastoma

The efficacy of the anti-CD70 IgG variant vcF4 conjugates was investigated in renal cell cancer xenograft models, 786-O and UMRC3, and the glioblastoma xenograft model, DBTRG05-MG (Fig. 4). ADCs were administered i.v. at two different dose levels into nude mice bearing established (~90 mm³) s.c. tumors, with the aim of achieving suboptimal dosing to discern any differences in ADC activity.

The single dose efficacy of the anti-CD70 variant vcF4 conjugates were first compared in the 786-O model and IgG1v1-vcF4 had the most potent antitumor activity (Fig. 4A). IgG1v1-vcF4 was estimated to be ~3-fold more potent than IgG1-vcF4 by comparison of the 0.5 and 1.5 mg/kg dose groups. The greater activity of IgG1v1-vcF4 over IgG1-vcF4 reached statistical significance at the 1.5 mg/kg dose (P = 0.0002) but not the 0.5 mg/kg dose (P = 0.15). IgG2-vcF4 showed antitumor activity intermediate between that of the IgG1-vcF4 parent and IgG1v1-vcF4 at
both dose levels, which reached statistical significance compared with IgG1-vcF4 only at the 1.5 mg/kg dose ($P = 0.039$). IgG4-vcF4 and IgG4v3-vcF4 showed slightly lower activity than IgG1-vcF4.

The single dose efficacy of the anti-CD70 variant vcF4 ADCs was then explored in the DBTRG05-MG model (Fig. 4B). IgG1v1-vcF4 had the most potent antitumor activity at the lower dose level (3.0 mg/kg) that approached statistical significance versus IgG1-vcF4 ($P = 0.054$), whereas the other anti-CD70 conjugates showed lower activity that was similar to each other. All ADCs achieved complete responses at the higher dose level (10 mg/kg), albeit with tumor outgrowth starting at $\sim$60 days after implantation.

IgG1-vcF4, IgG1v1-vcF4, and IgG2-vcF4 were selected for a multiple-dose efficacy (every 4 days times 4) comparison in a more challenging model (UMRC3). Consistent with single-dose efficacy studies, IgG1v1-vcF4 had the most potent antitumor activity at both 3.0 and 6.0 mg/kg dose levels, albeit without reaching statistical significance versus IgG1-vcF4. IgG1-vcF4 and IgG2-vcF4 showed lower activity that was similar to each other. Thus, IgG1v1-vcF4 shows improved antitumor activity over the parent IgG1-vcF4 conjugate in three different xenograft models that reached significance ($P < 0.05$) in the 786-O model and approached significance ($P = 0.054$) in the DBTRG05-MG model (Fig. 4A-C).

Next, we explored the effect of the linker between drug and antibody on the performance of ADCs using the anti-CD70 IgG variants. IgG1, IgG1v1, and IgG2 variants were conjugated to the nonprotease cleavable linker, maleimidocaproyl-MMAF (7) for evaluation in the 786-O model. The pattern of antitumor activities with the mcF4 conjugates (Fig. 4D) was qualitatively similar to that observed for the vcF4 ADCs (Fig. 4A). IgG1v1-mcF4 induced significantly improved tumor growth delays relative to the parent IgG1-mcF4 conjugate at the 1.5 mg/kg dose level ($P = 0.020$) and was also most potent at the 4.5 mg/kg dose level albeit without reaching statistical significance. IgG2-mcF4 showed antitumor activity intermediate between IgG1-mcF4 and IgG1v1-mcF4 at both dose levels that did not reach statistical significance. The nonconjugated anti-CD70 IgG1 and nonbinding control ADCs showed little to no antitumor activity in all of the solid tumor xenograft models (Fig. 4).

**Maximum Tolerated Dose**

ADC maximum tolerated dose in BALB/c mice was defined as the highest dose that did not induce $\geq 20\%$ weight loss or severe signs of distress. The anti-CD70 antibody, h1F6, binds to human CD70 but does not cross-react with the corresponding antigen from mice. Thus, antigen-independent but not antigen-dependent toxicities of the anti-CD70 ADCs can be explored in mice. All ADCs were tolerated to $\geq 40$ mg/kg with IgG2-vcF4 being slightly better tolerated ($\geq 60$ mg/kg) (Table 2). Thus, from the efficacy data (Fig. 4) and tolerability data (Table 2), IgG1v1-vcF4 has a therapeutic index that is improved $\sim 2$-fold compared with the parent conjugate, IgG1-vcF4.

**Discussion**

At least 10 different ADCs are in oncology clinical trials as of May 2008, including conjugates constructed from antibodies of IgG1, IgG2, and IgG4 isotypes (3). Surprisingly, no preclinical comparison of the efficacy and tolerability of ADCs of different isotypes has been reported previously. This motivated us to undertake such an analysis using IgG1, IgG2, and IgG4 isotype variants of a humanized anti-CD70 antibody conjugated to the potent cytotoxic auristatin drug, MMAF (7). Conjugation was done via solvent accessible cysteines of which there are 8 such residues in IgG1 and IgG4 and 12 in IgG2. An IgG3 variant was not constructed as we are unaware of any nonconjugated IgG3- or IgG3-based ADCs in clinical trials. The 22 solvent accessible cysteine residues in IgG3 would add much complexity to ADC generation and analysis.
Mean drug/antibody ratio appears to be a more important determinant of ADC potency and tolerability in vivo than conjugate homogeneity or sites of drug attachment (13–15). The mean drug/antibody ratio was therefore fixed within a narrow range for ADCs in this study. All IgG variants were partially reduced with TCEP and then reproducibly conjugated to mc-vcMMAF with a mean stoichiometry of ∼4 drugs/antibody (Table 1). This choice of stoichiometry reflects experience with the chimeric IgG1 antibody, cAC10, conjugated to a closely related auristatin, mc-vcMMAE, where a 2- to 3-fold improvement in therapeutic index was achieved by reducing the stoichiometry from 8 to 2 or 4 drugs/antibody (15).

A priori, ADC interactions with Fcγ receptors might be advantageous by endowing them with additional antitumor activity such as ADCC. Conversely, ADC binding to effector cells might reduce their localization to tumors or impair their internalization by target cells or give rise to additional off-target toxicities. IgG2 and IgG4 have lower affinity for huFcγRI than do IgG1 (27). Additional IgG variants were generated to investigate the influence of interactions with Fcγ receptors on ADC properties more directly. The anti-CD70 IgG1 bound to huFcγRI and huFcγRIIIA V158, and these interactions were greatly attenuated in IgG1v1 containing the mutations, E233P:L234V:L235A (Fig. 1A), as anticipated (9, 22). The IgG4 variant bound to huFcγRI, but not to huFcγRIIIA V158, and this interaction was virtually eliminated by the mutations S228P:L235A:G237A:E318A, as anticipated (10). The IgG1 parent, but not IgG2 or IgG4, competed for binding to the murine receptor, muFcγRIV, and this interaction was abolished in the IgG1v1 variant (Fig. 1A).

Drug conjugation had no measurable effect on binding of the IgG1 parent to huFcγRI, huFcγRIIIA, and muFcγRIV in competition assays and similarly did not alter IgG4 binding to huFcγRI (Fig. 1A). This maintained ability of IgG1-vcF4 and IgG4-vcF4 ADCs to bind one or more Fcγ receptors reflects in part the variable stoichiometry of drug loading, including the presence of unmodified antibody (e.g., the chimeric IgG1, cAC10, conjugated by the TCEP method to a mean stoichiometry of 4 equivalents of mc-vcMMAE per antibody was a mixture with 0, 2, 4, 6, or 8 drugs/antibody in the approximate percentages of 9%, 22%, 36%, 24%, and 10%, respectively; ref. 14). Drugs were distributed across

![Figure 3. Pharmacokinetics of anti-CD70 IgG variant ADCs. Groups of nude mice (n = 3) were treated with 10 mg/kg ADCs and serum samples were collected and analyzed by ELISA to determine the total antibody concentration. Mean with unidirectional error bars + SE for clarity. Insert, data for the first 3 days of the experiment, corresponding to the α phase; main figure, data for the entire experiment, corresponding to the α and β phases of the biphasic disposition (Table 2).](image-url)

**Table 2. ADC pharmacokinetics and tolerability**

<table>
<thead>
<tr>
<th>Pharmacokinetic variables</th>
<th>IgG1-vcF4</th>
<th>IgG1v1-vcF4</th>
<th>IgG2-vcF4</th>
<th>IgG4-vcF4</th>
<th>IgG4v3-vcF4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal half-life (d)</td>
<td>5.4 ± 1.1</td>
<td>5.3 ± 0.7</td>
<td>5.9 ± 1.2</td>
<td>2.8 ± 0.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Clearance (mL/d/kg)</td>
<td>51 ± 5</td>
<td>23 ± 1</td>
<td>23 ± 5</td>
<td>61 ± 17</td>
<td>103</td>
</tr>
<tr>
<td>AUC_{0-∞} (d μg/mL)</td>
<td>200 ± 23</td>
<td>432 ± 19</td>
<td>469 ± 115</td>
<td>204 ± 74</td>
<td>97</td>
</tr>
<tr>
<td>α-phase contribution to AUC (%)</td>
<td>62 ± 8</td>
<td>36 ± 4</td>
<td>27 ± 2</td>
<td>60 ± 12</td>
<td>64</td>
</tr>
<tr>
<td>β-phase contribution to AUC (%)</td>
<td>38 ± 8</td>
<td>64 ± 4</td>
<td>73 ± 2</td>
<td>40 ± 12</td>
<td>36</td>
</tr>
<tr>
<td>Tolerability</td>
<td>≥40</td>
<td>≥40</td>
<td>≥60</td>
<td>≥40</td>
<td>≥40</td>
</tr>
</tbody>
</table>

**NOTE:** The single maximum tolerated dose in BALB/c mice was the highest dose that did not induce ≥20% weight loss or severe signs of distress in two independent experiments (three mice per group).

1Clearance, terminal half-life, and AUC_{0-∞} were estimated by noncompartmental analysis (mean ± SE, three mice per group). Data from one mouse for IgG4v3-vcF4 could not be successfully fit.

1For all IgG variant ADCs, compartmental analysis revealed a biphasic disposition with a transition from α to β phases at ∼3 days after dosing. The AUC from 0 to 3 and 3 to ∞ days were used to estimate the α- and β-phase contributions to AUC, respectively.
Figure 4. Single and multiple dose efficacy of anti-CD70 IgG variant ADCs against established tumor xenografts. 

A, vcF4 ADCs in the 786-O renal cell cancer model. 
B, vcF4 ADCs in the DBTRG05-MG glioblastoma model. 
C, vcF4 ADCs in the UMRC3 renal cell cancer model. 
D, mcF4 ADCs in the 786-O model. Groups of nude mice with ~90 mm³ s.c. tumors were dosed (arrows) by i.v. injection once for 786-O and DBTRG05-MG models or every 4 days times 4 for the UMRC3 model. There were 8 or 9 mice per group, except for the untreated group in A (n = 6). Mean tumor volume plots (shown) and median tumor volume plots (Supplementary Fig. S2) were very similar and were continued for each group until one or more animals died or was euthanized (see Materials and Methods). Representative of independent experiments. Mean ± SE tumor volumes corresponding to A to D are provided in Supplementary Tables S1 to S4, respectively.
eight potential attachment sites on solvent accessible cysteine residues (14). Using the analytical methods of Sun et al. (14), we estimate that roughly half of the antibodies in our ADC preparations contain at least one hinge disulfide (data not shown). Maintaining at least one of the two interheavy chain hinge disulfide bonds in a humanized IgG1 preserves efficient binding to huFcRIIIA as judged by Cys→Ser mutations (28). In contrast, replacement of both hinge cysteines with serines greatly reduces the ability to support ADCC presumably via impaired binding to huFcγR (29).

Several lines of evidence suggest that drug conjugation to IgG via solvent accessible cysteines is unlikely to impair binding to huFcγR by steric blockade. All eight cysteine residues potentially used for drug conjugation are distinct and separate from the Fc residues that contact the huFcγR revealed by detailed mutational mapping of a humanized IgG1 antibody (22). Additional evidence for the separation of the potential drug conjugation sites on an IgG1 and the huFcγR binding sites is provided by a molecular model of an IgG1-vcF8 ADC bound to huFcRIIB (Fig. 1D). IgG/Fcγ receptor interactions show a high degree of structural conservation (30), so observations with IgG1 and huFcRIIB seem likely to extend to other IgG isotopes, huFcγR and muFcγR.

Elevated antigen levels can lead to greater ADC potency in cytotoxicity assays in vitro as shown using transfected cell lines expressing varying levels of target antigen (31, 32). The basis for this enhanced potency is not well defined but may reflect greater ADC binding, internalization, and accumulation for cell lines expressing higher antigen levels. In contrast, for the anti-CD70 IgG variants described here, there was only limited correlation between the antigen expression levels and sensitivity to ADCs. For example, the three tumor cell lines expressing ≥100,000 copies of CD70 per cell showed slightly greater sensitivity to the ADCs than the three lines expressing lower levels of antigen (Fig. 2). This may reflect that tumor, but not transfected, cell lines differ in many ways that could potentially affect their sensitivity to ADCs in addition to antigen expression levels. Limited correlation between antigen expression and sensitivity to ADCs was reported previously by us for auristatin-containing ADCs targeting melanotransferrin/p97 (21).

The ADCs showed pharmacokinetic differences in mice (Fig. 3; Table 2) that are broadly consistent with data from a matched set of chimeric IgG comprising murine variable domains and human constant domains (ref. 26). For example, the ~2-fold greater terminal half-life of IgG1-vcF4 than IgG4-vcF4 (5.4 versus 2.8 days) is very similar to the chimeric IgG1 and IgG4 with terminal half-lives of 7.0 and 3.0 days, respectively (26). The AUC for IgG1v1-vcF4 and IgG2v2-vcF4 was about 2-fold higher than for IgG1-vcF4, correlating with greater β-phase contributions to the AUC (Table 2). Greater AUC was also shown previously for a chimeric IgG2 compared with the corresponding IgG1, although this was not quantified (26). We observed similar terminal half-lives for IgG1-vcF4, IgG1v1-vcF4, and IgG2-vcF4, whereas a ~2-fold longer half-life was reported for IgG2 than IgG1 (26). The concordance between this study and that of Zuckier et al. (26) is striking given the numerous differences between these studies: ADCs versus naked IgG, antigen-binding ELISA versus 125I detection, nude mice versus SCID mice, and 63- versus 8-day study duration, respectively.

The molecular basis for the observed pharmacokinetic differences for the ADCs remains to be determined. Possible explanations include altered interactions of the ADCs with muFcγRIV and other muFcγR, changes in binding to the salvage receptor, FcRn, or differences in the ADC in vivo stability. Binding of human IgG to mouse FcRn contributes to their long terminal half-life in mice as judged by pharmacokinetic experiments in wild type and FcRn-null mice (33). However, differences in terminal half-life of chimeric IgG of varying human IgG isotopes in mice did not correlate with any single binding variable for interaction with FcRn and terminal half-life (34). The shorter terminal half-life of IgG4-vcF4 compared with IgG1-vcF4 and IgG2-vcF4 in mice (Table 2) contrasts with man where endogenous human IgG1, IgG2, and IgG4 have a similar mean half-life of 21 days (35). Thus, murine xenograft studies may underpredict the clinical potential of IgG4-based ADCs.

IgG1v1-vcF4 had greater in vivo antitumor activity than IgG1-vcF4 in three different xenograft models that reached statistical significance in the 786-O renal cell cancer model (P = 0.0002) and approached statistical significance in the DBTRG05-MG glioblastoma model (P = 0.054). The nonconjugated IgG1 antibody had no antitumor activity in these solid tumor models, in contrast to its potent activity in hematologic tumor models (36). Thus, the antitumor activity of the ADCs in these solid tumor models is apparently due to the auristatin drug rather than activities of the antibody delivery vehicle such as effector functions. IgG1-vcF4 and IgG1v1-vcF4 were indistinguishable in their in vitro cytotoxic activity against cell lines corresponding to the xenograft models (Fig. 2), thereby eliminating one possible explanation for differences in their in vivo activities. The improved in vivo performance of IgG1v1-vcF4 over IgG1-vcF4 correlated with a 2-fold increase in AUC (Table 2), which likely provides greater tumor exposure to IgG1v1-vcF4. However, data from other ADCs suggest that pharmacokinetic differences are, at best, only a partial explanation for observed efficacy differences. For example, IgG2-vcF4 showed a modest but reproducible improvement in antitumor activity compared with IgG1-vcF4 in only one of three models (786-O; Fig. 4A), despite having a 2-fold greater AUC, similar to IgG1v1-vcF4.

The enhanced efficacy of IgG1v1-vcF4 over IgG1-vcF4 (Fig. 4) in conjunction with similar tolerability in mice (Table 2) suggests an improvement in therapeutic index. IgG1-vcF4, IgG1v1-vcF4, and IgG2-vcF4 had very similar toxicity profiles in an exploratory rat toxicology study as did IgG1-mcF4, IgG1v1-mcF4, and IgG2-mcF4 (data not shown). Attenuated FcγR binding does not appear to be a major factor in the improved antitumor activity and therapeutic index observed for IgG1v1-vcF4 compared with
IgG1-vcF4, because IgG2-vcF4 also has greatly impaired binding to muFcRIV with only modestly enhanced antitumor activity (Fig. 4). However, ADC binding to other muFcR remains to be explored. Further study is needed to elucidate the basis of the improved therapeutic index of IgG1v1-vcF4 over IgG1-vcF4.

The effect of antibody Fc mutations and isotype on ADC function is challenging to study preclinically because of differences between human and mouse immune systems, including Fcy receptors and FcRn, as well as the absence of endogenous IgG in immunocompromised mice typically used for xenograft studies. For ADCs targeting B cells, nonhuman primate studies are anticipated to allow assessment of B-cell ablation as a surrogate measurement for efficacy in addition to safety.

The IgG delivery vehicle modification strategy used here for enhancing ADC therapeutic index is potentially applicable to other antibodies as it is independent of the antigen-binding variable domains. Indeed, we are currently exploring this possibility along with the anticipated applicability to other drugs and linkers.

Disclosure of Potential Conflicts of Interest

All authors were employees of Seattle Genetics with ownership interests in the company at the time that this work was undertaken. No other potential conflicts of interest were disclosed.

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References

Molecular Cancer Therapeutics

Engineered anti-CD70 antibody-drug conjugate with increased therapeutic index

Charlotte F. McDonagh, Kristine M. Kim, Eileen Turcott, et al.


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