

Contribution of Linker Stability to the Activities of Anticancer Immunoconjugates

Stephen C. Alley, Dennis R. Benjamin, Scott C. Jeffrey, Nicole M. Okeley, Damon L. Meyer, Russell J. Sanderson, and Peter D. Senter*

Seattle Genetics, Inc., 21823 30th Drive SE, Bothell, Washington 98021. Received November 26, 2007;
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The linker component of antibody–drug conjugates (ADC) is a key feature in developing optimized therapeutic agents that are highly active at well tolerated doses. For maximal intratumoral drug delivery, linkers are required that are highly stable in the systemic circulation, yet allow for efficient drug release at the target site. In this respect, amide bond-based technologies constitute a technological advancement, since the linker half-lives in circulation ($t_{1/2} \sim 7$ days) are much longer than earlier generation linkers that break down within 1–2 days. The amide linkers, some of which contain peptides, are appended to the mAb carriers through thioether/maleimide adducts. Here, we describe that use of a bromoacetamidocaproyl (bac) in place of the maleimidocaproyl (mc) increases the plasma stability of resulting thioether ADCs. One such ADC, 1F6-C4v2-bac-MMAF, which is directed against the CD70 antigen on lymphomas and renal cell carcinoma, was prepared containing a bac thioether spacer between the drug (MMAF) and the mAb carrier (1F6-C4v2). There was no measurable systemic drug release from this ADC for 2 weeks postadministration in mice. In order to assess the impact of improving linker stability beyond mc containing ADCs, a series of mc and bac-linked 1F6-MMAF conjugates were compared for tolerability, intratumoral drug delivery, and therapeutic efficacy in nude mice with renal cell carcinoma xenografts. There were no statistically significant efficacy differences between sets of mc and bac containing ADCs, although the bac linker technology led to 25% higher intratumoral drug exposure over a 7 day period compared to the corresponding mc linker. The mechanism of drug release from maleimide-adducts likely involves a retro-Michael reaction that takes place in plasma, based on *in vitro* studies demonstrating that some of the released drug-maleimide derivative became covalently bound to cysteine-34 of serum albumin. In summary, the data indicate that new linkers can be obtained with improved *in vivo* stability by replacing the maleimide with an acetamide, but the resulting ADCs had similar tolerability and activity profiles.

INTRODUCTION

The toxicity associated with systemically administered anticancer drugs has generated significant interest toward improving drug delivery to tumors while sparing nontarget tissues from chemotherapeutic damage. Antibody–drug conjugates (ADCs), composed of cytotoxic agents fused via conditionally labile linkers to monoclonal antibodies (mAbs) against tumor associated antigens, have figured prominently within this field, with clinically approved drug such as Mylotarg (anti-CD33-calechemycin) for the treatment of acute myelogenous leukemia (1–3) and an array of newer ADCs in various stages of preclinical and clinical development (4–14). Studies surrounding these agents have led to the identification of several critical parameters that must be addressed in order to develop targeted drugs with broad applicability for clinical cancer medicine. These include nontarget tissue uptake of ADCs, the heterogeneity of both antigen expression and macromolecular uptake within tumor masses, inappropriate linker stability, inefficient drug release within tumors, and release of drugs with low potency (6, 15, 16).

Linker stability plays a central role in ADC efficacy and tolerability. Early generation ADC linkers were commonly derived from disulfides and acid-labile hydrazones, both of which were designed to be cleaved inside target cancer cells, but inevitably underwent cleavage at nontarget sites. Because of this, significant effort has been directed toward balancing the stability of these linkers in the circulation, while still allowing them to be sufficiently labile for activation at the target

site. One of the first systematic studies along these lines involved conjugates of ricin A-chain attached to mAbs through differentially hindered disulfide bonds (17, 18). The hindered disulfide provided improved conjugate stability *in vivo*, together with a better therapeutic window in xenograft tumors. These findings were later extended to include ADCs composed of the potent cytotoxin maytansine. Conjugates with sterically hindered disulfides displayed potent antitumor activity and had improved biological stability compared to their less hindered counterparts (6, 15, 16, 19). Consistent with this trend is our earlier finding that acid-labile hydrazone linkers provided lower specificity *in vitro* and higher levels of toxicity *in vivo* compared to highly stable linkers (20). Such studies, together with others (21, 22), demonstrate how linker stability can influence ADC activity, specificity, and toxicity.

The approach we have taken toward advancing conditionally labile linker technologies involves the use of intracellular enzymes for drug release. The first example involved valine-citulline (vc) dipeptide derivatives of the potent antimetabolic drug, monomethyl auristatin E (MMAE), which were designed to release MMAE upon exposure of the ADCs to lysosomal proteases such as cathepsin B (20, 23). While lysosomal enzymes can be detected in the blood, their activities are very low due to endogenous inhibitors and the high pH value of blood compared to lysosomes. Thus, free MMAE was released from vc-linked MMAE ADCs in plasma more slowly ($t_{1/2} > 30$ d) than corresponding acid-labile hydrazone-based ADCs ($t_{1/2} \sim 2$ d) (20). This trend in increased ADC stability was also observed *in vivo*, in that the half-life of drug release from the ADC was 6

* Corresponding author. E-mail psenter@seagen.com.

days in mice and 10 days in Cynomolgus monkeys for the vc-linked drug (24), which is considerably longer than previously reported half-lives of hydrazones and disulfides (24–26).

The effects of extending in vivo linker half-life beyond 6–10 days are not known. To investigate this, we describe the generation of new ADCs with drug linkers that show no degradation in the circulation for up to 14 days. Interestingly, this new drug linker does not impact ADC in vivo therapeutic efficacy and tolerability when compared with ADCs that have half-lives of approximately 7 days.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents. 1F6 (chimeric anti-CD70 (27)), 1F6-C4v2 (humanized anti-CD70 with cys to ser heavy chain amino acid substitutions at positions 226 and 229) (28) and AC10 (chimeric anti-CD30 (23)) are IgG1 mAbs that were isolated as previously described. The human renal cell carcinoma cell lines 786-O, UMRC-3, and Caki-1 were obtained from ATCC. The human lymphoma cell line L428 was obtained from the DSMZ (Braunschweig, Germany). All cell lines were cultured according to the manufacturers suggested protocol. LC/MS and LC/MS/MS spectra were recorded on a MicroMass Q-TOF Ultima mass spectrometer equipped with a Z-spray ion source and a Waters 2795 HPLC system. mc-MMAF, mc-vc-MMAF, bac-MMAF (LC-MS m/z (ES^+) 965.8 (M+H)), and bac-vc-MMAF (LC-MS m/z (ES^+) 1370.7 (M+H)) were obtained as previously described (29, 30).

Preparation of Conjugates. Alkyl-maleimide linked 1F6, 1F6-C4v2, and AC10 ADCs were prepared as previous described (28, 30). For the bromoacetamides, the reduced antibodies (30, 31) were adjusted to pH 8 with sodium borate and incubated with 2 equiv of bac-vc-MMAF or bac-MMAF per antibody cysteine for 4 h at 25 °C. Subsequent purification and characterization of all ADCs were as previously described (30). The conjugated antibodies were unimpaired with respect to affinity for their targets (CD70 for 1F6 and CD30 for AC10) and contained <2% aggregate and <0.5% unconjugated drug.

ELISAs. The concentration of the 1F6-C4v2 component of the ADCs in plasma was measured using a previously described ELISA assay (24) using 96-well plates coated with an anti-idiotypic-1F6 mAb at a concentration of 1 μ g/mL in 100 mM sodium carbonate/bicarbonate (pH 9.6). The plasma samples were then diluted to 500 ng/mL h1F6-C4v2 mAb into PBS with 0.05% Tween 20 containing 1% BSA (PBST/BSA) and added to wells in triplicate. An hour later, the wells were washed, and anti-MMAF mAb SG3.265-biotin (1 μ g/mL in PBST/BSA) was added and incubated an additional hour. The wells were washed, and streptavidin-HRP (diluted into PBST/BSA) was added and incubated for 1 h at room temperature. Detection was initiated using TMB substrate added to the washed wells. Reactions were terminated with 1 N H_2SO_4 and absorbance was measured at 450 nm against a reference wavelength of 630 nm. On the same 96-well microtiter plate, samples and standards were also subjected to total mAb ELISA analysis to ensure that the amount of mAb backbone captured to the plate were comparable. The results were compared with standards consisting of purified 1F6-C4v2-mc-MMAF_n or 1F6-C4v2-bac-MMAF_n ADC standards in plasma ($n = 1, 2, 3$, or 4 drug-linkers per mAb) that were similarly processed. Linear regression analysis of data from the standards was used to obtain an equation relating the average drug/mAb ratio of the relevant ADC to the measured OD₄₅₀.

Biodistribution. All animal studies were carried out in accordance with Animal Care and Use Committee guidelines. Balb/c nu/nu mice with subcutaneous 786-O tumors xenografts (in vivo passaged, 100–150 mm³) were treated i.v. with the ADCs at 1.5 mg/kg in PBS. At the time points indicated, mice were sacrificed by CO₂ inhalation, and were then terminally

bled. Serum, tumor, and liver were stored at –80 °C until all of the samples were collected. Tumor and liver samples were thawed on ice, 4 mL/gram of tissue of PBS was added, and the tissue was mechanically homogenized on ice. MMAF was extracted from 100 μ L homogenate by addition of 100 μ L of 0.2% formic acid in water and 400 μ L of acetonitrile. After spiking with ¹³C-MMAF internal standard (30), the mixture was incubated at 25 °C for 1 h with occasional vortexing. Following centrifugation to pellet cell debris and precipitated proteins, the supernatant was extracted using a Waters (Milford, MA) Oasis MCX 96-well plate that was conditioned with 0.4 mL (volumes are per well) of 2% ammonium hydroxide in dichloromethane: isopropyl alcohol (80:20), 0.4 mL of methanol, and 0.4 mL of water. The plate was washed with 0.4 mL of 0.2% aqueous formic acid and 0.4 mL of methanol. MMAF was eluted with 2 treatments of 0.4 mL of 2% ammonium hydroxide in dichloromethane/isopropyl alcohol (80:20). The elution solvent was evaporated in a vacuum concentrator and samples were reconstituted in 50 μ L of 50% aqueous acetonitrile. For serum samples, serum was mixed with 1 volume of 0.2% aqueous formic acid, spiked with ¹³C-MMAF, and extracted as described above for the tumor and liver samples.

Samples were analyzed by LC-MS/MS by injecting 20 μ L of the reconstituted sample onto a 2.1 $\times$ 30 mm Agilent (Santa Clara, CA) Zorbax SB C8 column running on a Waters Acquity liquid chromatograph (flow rate 1 mL/minute; column temperature 40 °C; gradient conditions isocratic 5% B (0.1% formic acid in acetonitrile) 95% A (0.1% aqueous formic acid) for 0.5 min, 0.05 min linear gradient to 70% B, isocratic for 0.15 min, 0.05 min linear gradient to 95% B, isocratic 95% B for 0.05 min. The eluted solvent was directed into a Waters Quattro Premier triple quadrupole mass spectrometer operating in multiple reaction monitoring mode. MMAF was analyzed using its 732 m/z parent ion and 700 m/z fragment ion, while the ¹³C-MMAF internal standard was analyzed using its 738 m/z parent ion and 706 m/z fragment ion. Standard curves were generated by spiking MMAF and ¹³C-MMAF internal standards into tissue homogenates or serum from untreated tumor-bearing animals which were then treated as described above. All data collection and sample analyses were performed using *MassLynx* version 4.0.

Identification of mc-MMAF-Modified Peptides in Plasma.

AC10-mc-MMAF was diluted with 50% citrated rat plasma to 5 mg/mL. Half of the reaction was removed and stored at –20 °C as a time zero reference, and the remaining mixture was placed at 37 °C for 5 days. The samples (50 μ L) were treated with 8 M urea in 0.5 M ammonium bicarbonate (1.8 mL), dithiothreitol (45 mM, 0.36 mL) was added, and incubation was carried out at 50 °C for 20 min, followed by cooling to room temperature and treatment with iodoacetamide (2.2 mol equiv per mole dithiothreitol) in the dark for 15 min. The samples were then diluted with water to a final urea concentration of 1.9 M, and trypsin (TPCK, Pierce) was added (final concentration 13 μ g/mL). After overnight incubation at 37 °C, the solutions were desalted on Pep-Clean C18 spin columns (Pierce), using seven columns for each reaction. The resins were prepared according to manufacturer's instructions and the samples were loaded in 100 μ L steps with each sample being reloaded three times to ensure complete capture of peptides. The resin was washed with 5% acetonitrile 0.5% TFA (2 $\times$ 200 μ L) and then eluted with 70% acetonitrile (2 $\times$ 20 μ L, 1 $\times$ 50 μ L). The elutions for each sample were combined and dried under centrifugal vacuum. The peptide residues were dissolved in acetonitrile (87.5 μ L) and diluted with PBS (663 μ L). Particulates were collected by centrifugation (16 000g, 5 min) and were redissolved in DMSO (20 μ L) and combined with the super-

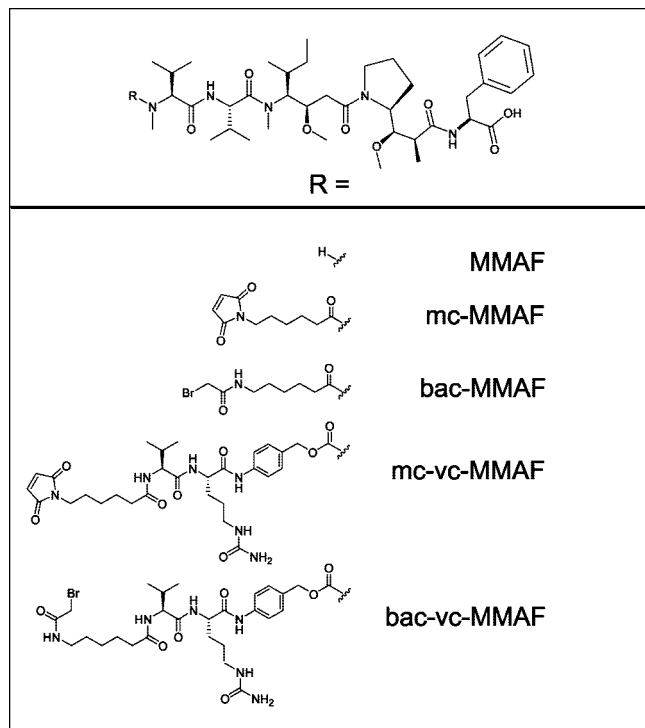


Figure 1. Structures of MMAF and linkers.

nantant. The samples were further diluted with additional PBS to bring the organic solvent composition to 6%.

An Affigel-10 resin coupled to the anti-mc-MMAF antibody SG2.15 was used to capture mc-MMAF-containing peptides from the samples. Each sample was added to 0.9 mL of resin bed and was mixed at 4 °C for 4 h. The slurries were washed with PBS (6 × 2 mL). The bound peptides were eluted by incubation with 5.5 M guanidine hydrochloride (Pierce) in PBS at room temperature for 4 h followed by 20 min at 37 °C. A second elution with fresh guanidine hydrochloride solution was carried out at 37 °C for 30 min. The combined guanidine elutions were desalted on Pep Clean C18 spin columns as previously described using three columns per sample. The eluted peptides were dried by centrifugal vacuum and dissolved in 0.1% TFA. Drug-captured and desalted peptides were examined by LCMS using a Poroshell 300SB-C18 column (Agilent, 2.1 × 75 mm, 5 μm). The samples were separated at 0.2 mL/minute on a gradient starting at 0% B (0.1% formic acid in acetonitrile), 100% A (0.1% formic acid) for 3 min, then ramping to 15% B over 7 min followed by 15–23% B over 30 min, 23–28% B over 1 min, 28–40% B over 10 min, and finally 40–100% B over 4 min.

In Vitro Cytotoxicity. Log phase cultures of cells were collected and cells were plated at seeding densities ranging from 500 to 10 000 cells/well according to predetermined conditions. After incubating for 24 h, serial dilutions of test molecules were added, and cultures incubated a further 4 days. Assessment of cellular growth was determined using Alamar Blue (Biosource International, Camarillo, CA) dye reduction as previously described (30). Briefly, a 40% solution (wt/vol) of freshly prepared Alamar Blue was added to the cells to constitute 10% culture volume. Cells were incubated for 4 h, and dye reduction was measured on a fluorescent plate reader.

In Vivo Efficacy and Tolerability. In vivo efficacy of ADCs were evaluated in female nude mice bearing 786-O xenografts. Once tumors grew to approximately 100 mm³, test articles in PBS were injected into the tail vein at the indicated doses. Tolerability of ADCs were evaluated using BALB/c mice. The maximum tolerated dose (MTD) is defined as the highest dose

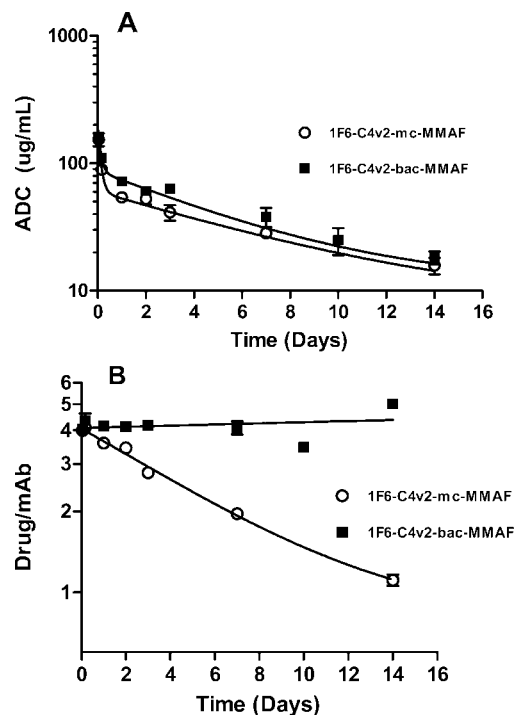


Figure 2. In vivo stability of auristatin ADCs. The mAb concentrations (A) were determined in plasma samples after a single injection of 10 mg/kg of 1F6-C4v2-mc-MMAF and 1F6-C4v2-bac-MMAF (3 animals/group). The drug/mAb ratios (B) were determined using an ELISA whose total signal is proportional to the drug loading of the ADC. The mc-MMAF conjugate shows dissociation of MMAF from the antibody with a half-life of approximately 7 days.

Table 1. IC₅₀ Values of Immunoconjugates on CD70 Expressing Cell Lines

ADC	IC ₅₀ (nM)				
	786-O	Caki-1	UMRC-3	L428	LP-1
1F6-C4v2-mc-MMAF	>1000	93	>1000	180	200
1F6-C4v2-bac-MMAF	450	90	490	52	88
1F6-C4v2-mc-vc-MMAF	23	32	87	10	71
1F6-C4v2-bac-vc-MMAF	29	35	37	13	70

Table 2. Percent Weight Change in Treated BALB/c Mice

ADC	day 4	day 9
1F6-C4v2-mc-MMAF 150 mg/kg	3.3	2.0
1F6-C4v2-bac-MMAF 150 mg/kg	−2.6	2.1
1F6-C4v2-mc-vc-MMAF 20 mg/kg	−5	3.0
1F6-C4v2-mc-vc-MMAF 40 mg/kg	−13	not tolerated
1F6-C4v2-mc-vc-MMAF 60 mg/kg	−19	not tolerated
1F6-C4v2-bac-vc-MMAF 20 mg/kg	−9	1.6
1F6-C4v2-bac-vc-MMAF 40 mg/kg	−19	not tolerated
1F6-C4v2-bac-vc-MMAF 60 mg/kg	−22	not tolerated

that did not induce >20% weight loss, distress, or overt toxicities in any of the animals, and was generally within 20% of doses where such events took place.

RESULTS

Auristatin Conjugates. The structures of the previously reported alkyl-maleimide containing drugs vc-MMAF and mc-MMAF (30), together with a set of related MMAF derivatives containing bromoacetamides in place of the alkyl-maleimide moieties, are shown in Figure 1. These MMAF-linker derivatives were designed to react with thiols, such as mAb cysteines generated after disulfide bond reduction. The antibodies used were 1F6 (chimeric IgG1 recognizing the CD70 antigen) (27), 1F6-C4v2 (humanized IgG1 anti-CD70 mAb with heavy chain

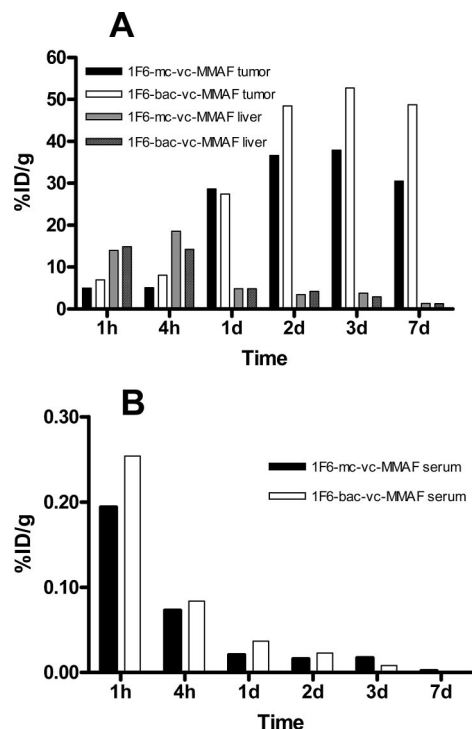


Figure 3. Concentrations of MMAF in (A) tumors and liver, and (B) serum obtained with mc-vc-MMAF and bac-vc-MMAF ADCs (2 animals/group). The results are expressed as the % of the injected dose (%ID) per gram of tissue. The initial weights of the tumors, livers, and blood were approximately 0.1, 1.0, and 1.0 g, respectively.

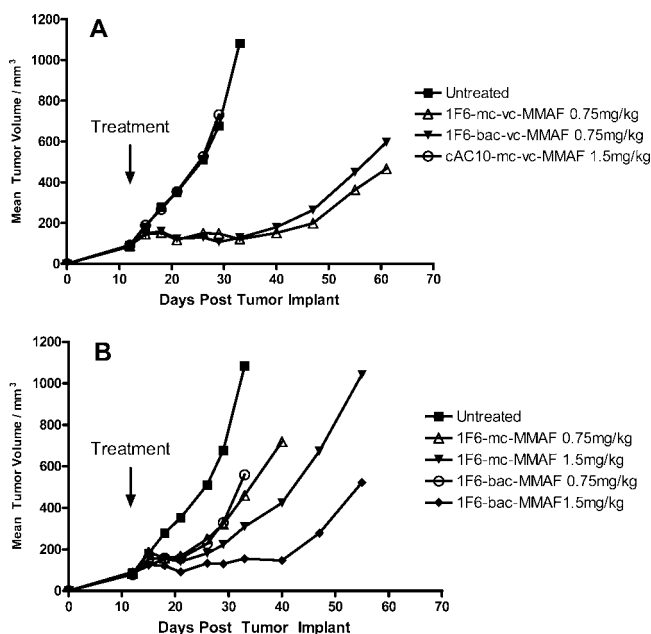


Figure 4. In vivo therapeutic efficacy of the conjugates in SCID mice. Groups of mice (five/group) with subcutaneous 786-O renal cell carcinoma xenografts were treated once (arrow) with conjugates at the doses indicated once the tumors were staged and growing. (A) Comparison of in vivo therapeutic efficacy of vc containing conjugates formed through acetamide (bac) and maleimide (mc) adducts, and (B) similar studies with directly linked ADCs lacking the vc peptide.

cysteine to serine substitutions at positions 226 and 229) (28), and AC10 (chimeric IgG1 recognizing the CD30 antigen) (23). Reduction of interchain disulfides was accomplished using the reducing agent tris(2-carboxyethyl)phosphine (TCEP), such that an average of two disulfides/mAb were reduced for the 1F6 and AC10 mAbs. Addition of the drug linker complexes led to

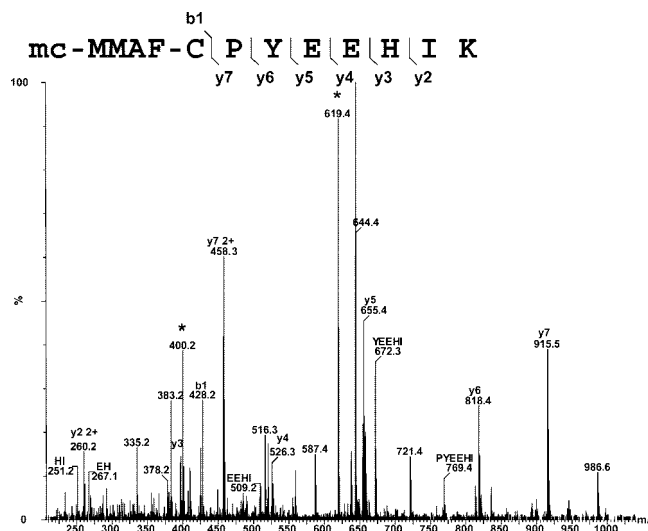


Figure 5. LC/MS/MS spectrum of the rat serum albumin (RSA) peptide-mc-MMAF adduct obtained upon incubation of AC10-mc-MMAF in rat plasma at 37 °C. The m/z of this peptide (648.4, (m+3)/3) and the MS/MS spectrum are consistent with RSA tryptic peptide T6 (residues 34–41 of the mature protein) modified with mc-MMAF at cysteine-34. Asterisks mark the a1 (400.2) ion containing mc-MMAF and the C-terminal tetrapeptide of MMAF (619.4), which is also a prominent in-source fragmentation ion.

conjugates having an average of four drugs/mAb, with a distribution centered at four drugs/mAb, but ranging between zero and eight. In contrast, the 1F6-C4v2 mAb provided ADCs that were highly homogeneous, since full reduction of the mutated mAb provided only four thiol groups for conjugation (28), which led to high yields of 1F6-C4v2-mc-MMAF with four drugs/mAb.

A previously described ELISA assay was used to determine drug to antibody ratios upon prolonged in vitro ADC incubation or after in vivo ADC administration (24). The results were compared with a 1F6-C4v2-bac-MMAF conjugate that did not contain a maleimide/thioether adduct. The recombinant mutated mAb was used for all these studies, ensuring that the administered drug was as homogeneous as possible, with initial uniform substitution of four drugs/mAb.

The first step in measuring in vivo ADC stability involved determining ADC concentrations in blood samples of treated animals. An anti-idiotypic-based ELISA provided a half-life for clearance of both conjugates in nude mice of approximately 7 days (Figure 2A). Samples from this assay were then subjected to an ELISA using an anti-drug mAb that bound to conjugated drug to an extent that reflected the level of drug substitution on the 1F6 mAb. As shown in Figure 2B, 1F6-C4v2-mc-MMAF lost drug with a half-life of 7 days, which is consistent with an earlier reported half-life of 6–10 days for a maleimide containing auristatin conjugate (24). In contrast, the 1F6-C4v2-bac-MMAF conjugate was more stable in vivo, with no apparent drug loss throughout the 14 day assay period.

Cytotoxicity. The 1F6-C4v2 auristatin conjugates were tested on a panel of CD70 expressing renal cell carcinoma cell lines, as shown in Table 1. Cells were exposed to the ADCs continuously for 96 h, and the cytotoxic effects, compared to untreated control cells, were determined by Alamar Blue conversion. Both the dipeptide ADCs with cleavable linkers (bac-vc and mc-vc-MMAF ADCs) exhibited potent and uniform activities against all cell lines tested. The fact that the two conjugates appear to be equally potent is not surprising, given that they release the same drug (MMAF) upon linker cleavage. In contrast, drug release from the 1F6-C4v2-mc-MMAF and bac-MMAF conjugates requires antibody degradation (30), and

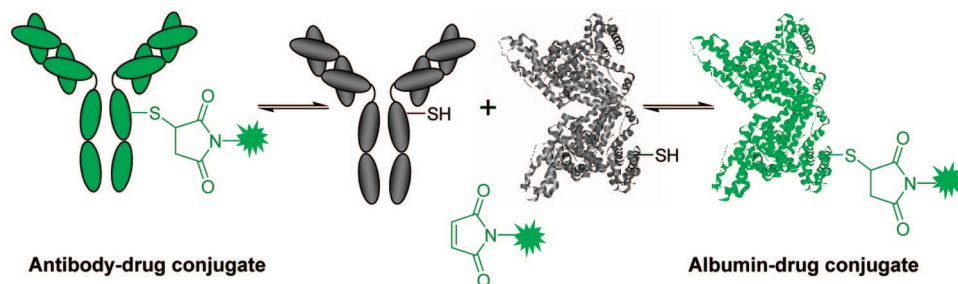


Figure 6. Proposed mechanism of drug transfer from an antibody to albumin through the thioether fragmentation reaction.

the released drugs are not chemically identical, since they contain linker and amino acids from the mAb. Based on previous studies of related ADCs (30), the 1F6-C4v2-mc-MMAF conjugate releases cysteine-mc-MMAF upon antibody degradation, and 1F6-C4v2-bac-MMAF should release the corresponding acetamidocysteine adduct. The data in Table 1 show that 1F6-C4v2-bac-MMAF leads to slightly greater potency than 1F6-C4v2-mc-MMAF on all but the Caki-1 cell line, and both ADCs are significantly less potent than the corresponding vc-MMAF ADCs.

Maximum Tolerated Doses. BALB/c nude mice were injected with the conjugates as indicated in Table 2, and the MTDs were determined based on body weight loss and outward signs of toxicity. Both 1F6-mc-MMAF and 1F6-bac-MMAF ADCs were nontoxic at 150 mg/kg, the highest dose tested. On the other hand, the dipeptide-containing ADCs, 1F6-mc-vc-MMAF and 1F6-bac-vc-MMAF, had MTDs of approximately 20 mg/kg. At 40 mg/kg, both ADCs were not tolerated. Therefore, tolerability was not apparently affected when comparing corresponding maleimide and acetamide derived ADCs. The differences in MTDs between the mc and vc-derived ADCs are most likely due to structural differences, as indicated above, between the released drugs.

Drug Delivery. An LC/MS/MS based assay was established to determine the amount of drug present in subcutaneous 786-O renal cell carcinoma xenografts, as well as the liver and serum of BALB/c mice treated with 1F6-mc-vc-MMAF and 1F6-bac-vc-MMAF conjugates at 1.5 mg/kg. The only identifiable drug released from these ADCs was MMAF, consistent with dipeptide cleavage followed by fragmentation of the *p*-aminobenzyloxycarbamate spacer (20). Figure 3A shows that tumor drug levels peaked at approximately 3 days, at which point 1F6-bac-vc-MMAF treated mice had higher intratumoral drug concentrations than those treated with the 1F6-mc-vc-MMAF ADC. Over the period of 7 days, the AUC difference was 25%. The liver (Figure 3A) and serum (Figure 3B) drug concentrations from the two ADCs were similar, consistent with early ADC exposure and lack of drug retention.

Efficacy. The therapeutic effects of the ADCs were determined in nude mice with subcutaneous 786-O tumor xenografts. Since the goal of these experiments was to determine if there were differences between the ADCs, each animal was injected only once at doses that were previously shown to be subcurative (27). As expected, 1F6-mc-vc-MMAF displayed significant antitumor activities (Figure 4A). Similar therapeutic effects were obtained with the corresponding 1F6-bac-vc-MMAF ADC, suggesting that the two agents were comparable in activity and potency. The antigen-specific nature of these activities was confirmed through the lack of activity displayed by cAC10-mc-vc-MMAF, an anti-CD30 ADC that does not bind to CD30 antigen negative 786-O cells.

A similar trend of activities was obtained with the ADCs that lacked the dipeptide spacer (Figure 4B). At 0.75 mg/kg, there was a slight delay in tumor outgrowth for both 1F6-mc-MMAF and 1F6-bac-MMAF with no apparent differences between the

two groups. The effects at 1.5 mg/kg were more pronounced for both ADCs. Although 1F6-bac-mc-MMAF appears to display a higher degree of antitumor activity than 1F6-mc-MMAF, the difference is not statistically significant (*P* values of 0.10 and 0.11 for days 47 and 55, respectively). These results are consistent with the localization studies which showed comparable levels of drug accumulation for bac and vc-based ADCs (Figure 3A).

Fate of Released Drug. Having established that linker plasma stability was enhanced upon replacement of the maleimide with an acetamide functionality, *in vitro* experiments were undertaken to identify the fate of the released drug from AC10-mc-MMAF. The ADC was incubated in rat plasma at 37 °C for 5 days, treated with trypsin, and auristatin-conjugated peptide fragments were separated from other plasma proteins by affinity chromatography on a column with an immobilized anti-auristatin mAb. Bound material was eluted, and the resulting peptides were analyzed by LC-MS. The identity of auristatin adducts was facilitated by the prominent *in-source* auristatin fragmentation peak at *m/z* 619.4. Before incubation, all of the auristatin adducts were comprised of antibody peptides that contained interchain cysteines. Comparison of these traces from the samples obtained after the 5 day plasma incubation revealed a new peak, whose *m/z* and MS/MS spectrum were consistent with a rat serum albumin peptide (residues 34 to 41) modified with the mc-MMAF drug linker (Figure 5). The fragmentation pattern of this peak demonstrated that mc-MMAF was covalently bound to cysteine-34 in albumin. We have confirmed that the same transfer reaction takes place in mice treated with 1F6-C4v2-mc-MMAF (data not shown). Thus, prolonged exposure of alkyl-maleimide auristatin ADCs to plasma proteins may lead to a slow transfer of drug to albumin cysteines. We propose that the mechanism for drug transfer proceeds through the reaction scheme depicted in Figure 6. The first step involves a reversible fragmentation reaction in which the alkyl-maleimide drug product dissociates from the mAb. In buffers such as PBS, this reaction is not observed, since the conjugation reaction is favored. However, in blood or plasma the predominant thiol-containing protein is albumin, which contains a reactive cysteine residue at position 34. The reaction of the maleimide with this cysteine results in the new species observed. The bac-containing ADCs are unable to undergo an analogous fragmentation reaction, and this accounts for their increased plasma stability.

DISCUSSION

Linker stability plays an important role in both the efficacy and toxicity of ADCs. Early generation ADCs often contained unstable linkers with short half-lives (1–2 days) such as disulfides (17, 18, 26) and hydrazones (19, 25, 32). More recently, attention has turned toward linkers with improved stability characteristics while in the systemic circulation (15, 30). Included among them are peptides (20, 33), glucuronides (34), and nonmetabolizable spacers that remain covalently attached to the drug after the mAb carrier is hydrolyzed in lysosomes of

target cells (15, 30). ADCs incorporating such linkers were formed through the reaction of thiols on one of the components with alkyl-maleimides on the other. In the examples presented here, 1F6-C4v2-mc-MMAF and 1F6-C4v2-mc-vc-MMAF were both stable in buffers, but slowly released drug with approximately 7 day half-lives upon exposure to plasma proteins in vitro and in vivo. We demonstrate that simply by replacement of the maleimide with an acetamide, ADC stability can be significantly increased. There was no apparent drug release from circulating 1F6-C4v2-mc-MMAF in vivo 14 days after administration (Figure 2B). However, in spite of the stability differences between the bromoacetamide and alkyl-maleimide based ADCs, their efficacy, potency, and toxicity characteristics were indistinguishable. This is most likely due to the very similar half-lives (approximately 7 days) of both drug dissociation from the alkyl-maleimide linked conjugates and ADC blood clearance (Figure 2). Extending linker half-life much beyond the pharmacokinetic half-life is not likely to have a pronounced effect on drug exposure, due to reduced ADC concentrations over time. We have calculated a maximum blood AUC difference between the acetamides and maleimide/thioether adducts over 7 days of only 30%, which is in agreement with the 25% difference in tumors shown in Figure 3A. The relative effects on therapeutic efficacy resulting from such small differences are likely to be quite subtle.

The results reported here provide the first direct evidence that maleimide/thioether fragmentation can lead to conjugate instability. The results are similar to a previous report in which a bis-maleimide-linked radiometal immunoconjugate was shown to be unstable in serum, with transfer of the radiometal to albumin taking place (35). It was not determined whether the transfer reaction involved maleimide transfer or the formation of noncovalent albumin adducts. Here, we demonstrate that alkyl-maleimides undergo slow dissociation leading to covalent drug transfer to the cysteine-34 residue on serum albumin, the same residue that is thought to be alkylated when maleimide-containing anticancer drug derivatives are systemically administered (36–39). The transfer mechanism described here most likely involves regeneration of the maleimide through thioether fragmentation, followed by alkylation of the cysteine thiol group on albumin. Experiments to confirm the proposed mechanism include the lack of transfer to S-alkylated albumin, or lack of transfer to albumin in the presence of large excesses of competing thiols (data not shown).

In conclusion, we have demonstrated that ADCs with linker half-lives in the range of 7 days in mice result in nearly optimal safety and efficacy profiles. Attachment of the drug/linker derivatives to mAb thiols through acetamides extends the half-lives significantly by circumventing maleimide transfer. We are currently engaged in studies to more fully explore the activity and toxicity profiles associated with newer generation linker technologies, such as those described here.

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