Development of novel quaternary ammonium linkers for antibody-drug conjugates


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Abbreviations: ADCs = antibody-drug conjugates; AE = auristatin E; DAR = drug-to-antibody ratio; DMSO = dimethylsulfoxide; DMTub = N-desmethyltubulysin; EDTA = ethylenediaminetetraacetic acid; EGTA = ethylene glycol tetraacetic acid; FBS = fetal bovine serum; FITC-MMAF = fluorescein isothiocyanate-monomethylauristatin F; FP = fluorescence polarization; mAb = monoclonal antibody; mDPR = maleimidodiaminopropionic acid; MMAE = monomethylauristatin E; MWCO = molecular weight cut off; PBS = phosphate-buffered saline; PIPES = pipermazine-N,N'-bis(2-ethanesulfonic acid); PK =
pharmacokinetic; SBT = sheep brain tubulin; TCEP = tris(2-carboxyethyl)-phosphine; Tub = N\textsuperscript{14}-desacetoxytubulysin H

Abstract

A quaternary ammonium-based drug-linker has been developed to expand the scope of antibody-drug conjugate (ADC) payloads to include tertiary amines, a functional group commonly present in biologically active compounds. The linker strategy was exemplified with a \(\beta\)-glucuronidase-cleavable auristatin E construct. The drug-linker was found to efficiently release free auristatin E (AE) in the presence of \(\beta\)-glucuronidase and provide ADCs that were highly stable in plasma. Anti-CD30 conjugates comprised of the glucuronide-AE linker were potent and immunologically specific \textit{in vitro} and \textit{in vivo}, displaying pharmacological properties comparable to a carbamate-linked glucuronide-monomethylauristatin E control. The quaternary ammonium linker was then applied to a tubulysin antimitotic drug that contained an N-terminal tertiary amine that was important for activity. A glucuronide-tubulysin quaternary ammonium linker was synthesized and evaluated as an ADC payload, in which the resulting conjugates were found to be potent and immunologically specific \textit{in vitro}, and displayed a high level of activity in a Hodgkin lymphoma xenograft. Furthermore, the results were superior to those obtained with a related tubulysin derivative containing a secondary amine N-terminus for conjugation using previously known linker technology. The quaternary ammonium linker represents a significant advance in linker technology, enabling stable conjugation of payloads with tertiary amine residues.
Introduction

Antibody-drug conjugates (ADCs) have emerged as a therapeutic modality for cancer as evidenced by the clinical success of brentuximab vedotin for relapsed/refractory Hodgkin lymphoma (1) and ado-trastuzumab emtansine for relapsed HER2+ breast cancer (2). Advances in linker technology played a role in those successes, enabling payload conjugation, pronounced circulation stability, and facile drug release upon antigen binding and intracellular uptake. However, linker technologies remain limited in scope, since drugs that are conjugated must contain certain reactive functional groups amenable to existing drug-linker chemistries (3).

An effective drug-linker for ADC-based delivery consists of multiple molecular components working in concert to achieve circulation-stability and rapid drug release upon antigen-mediated internalization. The key components of a cleavable linker often include a reactive group such as a maleimide or active ester for covalent linkage to the targeting antibody, a conditionally-stable release mechanism (e.g. disulfides, hydrolytically-labile groups, peptides, and glucuronides), and a self-immolative spacer configured for attachment to the cytotoxic payload (4, 5). Central to this strategy is the availability of a suitable functional group in the payload for attachment of the linker. Commonly used functional groups for linker attachment include primary (6, 7) and secondary amines (8), phenols (9), and sulfhydryls (10). A variety of linker chemistries based on these functional groups have been developed in recent years (4, 5).

The tertiary amine functional group is a common structural motif present in many biologically active molecules, but has not been utilized as a linker element in previously described ADCs for cancer therapy. To address this limitation, it has either been necessary to synthetically generate analogues with secondary amines, or to conjugate through other functional groups present within the drug structure. For example, auristatin E (1, Figure 1), a representative member of the potent dolastatin...
family of antimitotic drugs (11), was not amenable to conventional conjugation technologies, prompting the synthesis of a secondary amine containing analogue, monomethylauristatin E (MMAE, 2, Figure 1). The secondary amine was then conjugated to mAbs with a carbamate-based, proteolytically-labile drug-linker (8). The favorable characteristics of this drug-linker combination eventually led to the warhead component of the approved ADC, brentuximab vedotin (1).

Unfortunately, it is not always possible to maintain drug potency in cases where the tertiary amine plays an integral role in drug activity. This is exemplified by the tubulysins (12), a potent antimitotic drug class that binds tubulin with high affinity in the vinca site (13). The tubulysins are a compelling cytotoxic drug class for antibody conjugation due, in part, to their high potencies in the presence of multidrug resistance (MDR)-conferring transporter proteins (14). However, modification of tubulysins to introduce a functional group for conjugation is commonly not well tolerated. For example, the N-terminal N-desmethyl analogue of a keto-tubulysin derivative was found to be 100-fold less potent than the tertiary amine-containing parent (15). Similarly, conversion of N14-desacetoxytubulysin H (hereafter referred to as tubulysin analogue or Tub 3) to the desmethyl analogue DMTub 4 to reveal a readily conjugatable secondary amine resulted in a greater than a 30-fold drop in cellular and biochemical potency as a free drug (vide infra). Derivatization of the C-terminus through conversion of the carboxylic acid to a hydrazide (16) or modification of the tubuphenylalanine ring (17) appears more promising. However, these changes attenuate potency relative to the unmodified parent tubulysin.

The goal of this work was to exemplify the quaternary ammonium linker strategy (Figure 1B) with drugs such as auristatin E (1) and the tubulysin analogue 3, both of which are highly potent and have great potential as ADC cytotoxic warheads. Here, we demonstrate for the first time that potent cytotoxic anticancer drugs can be conjugated to mAbs through a tertiary amine functionality, and that the resulting ADCs are stable under physiologic conditions, highly active in vitro and in vivo,
immunologically specific. The results expand the repertoire of drugs that can be utilized within ADCs for targeted drug delivery to cancer cells.

**Materials and Methods**

**Preparation of ADCs bearing quaternary ammonium linkers**

The drug linkers shown in Figure 2 were synthesized from previously described intermediates (18, 19) and commercially available reagents as described in the Supplementary Information. Antibody-drug conjugates loaded at 8-drugs/mAb were prepared by treating the mAb at approximately 10 mg/ml in PBS (pH 7.4) containing 1 mM EDTA with 12 equivalents of tris(2-carboxyethyl)-phosphine (TCEP) to achieve full reduction of the four native interchain disulfides. Reduction progress was monitored by reversed-phase chromatography and additional TCEP was added as needed to complete the reaction. The reduced mAb was purified by ultrafiltration (three rounds, ten-fold dilution, centrifugation at 4,000 g through a 30 kDa MWCO filter).

Fully reduced mAbs in PBS containing 1 mM EDTA and buffered with 100 mM potassium phosphate to pH 7.4 were conjugated with 10 molar equivalents (25% excess) of drug-linker as a 10 mM DMSO stock. The resulting solution was vortexed and left at room temperature for 15-30 minutes. The extent of conjugation was assessed by reversed-phase chromatography and additional drug-linker was added as needed. Once all available mAb cysteines were alkylated, the crude ADC solution was purified by buffer-exchange into PBS using a NAP-5 desalting column (GE Healthcare, Chicago, IL) followed by ultrafiltration. The extent of aggregation was assessed by size exclusion chromatography, and in all cases the ADCs were found to be >95% monomeric. The final ADC concentration was measured spectrophotometrically and the resulting ADCs (8-drugs/mAb) were sterile-filtered through a 0.22 µm centrifugal filter and stored at -80 °C. For 4-loaded conjugates, the reduction step was carried out with
sub-stoichiometric TCEP to achieve partial reduction, revealing on average 4-thiols per mAb, and the subsequent conjugation, purification, and analysis was carried out as previously described.

**Enzymatic drug release experiments**

A 15 µl aliquot of 10 mM drug-linker stock of 6 or 7 in dimethylacetamide was added to 27 µl of dimethylsulfoxide and quenched with N-acetylcysteine (30 µl of 100 mM stock in PBS) in an Eppendorf tube. PBS was then added (48 µl) and the tube was allowed to sit at room temperature for 1 hour.

Enzyme stocks were prepared concurrently by dissolving β-glucuronidase (from bovine liver, ≥1,000,000 units/g solid) in 100 mM NaOAc (pH 4.7) to a final concentration of 0.5 or 1 mg/ml. The quenched linker solution (38 µl) was combined with 170 µl of fresh enzyme stock and 132 µl of 100 mM NaOAc (pH 4.7) then incubated at 37 °C. At each time point, 25 µl was withdrawn and added to 150 µl methanol, sealed and stored at -80 °C until all time points were taken. Samples were then centrifuged at high speed for 20 minutes and analyzed by UPLC-MS, counting ions for intact quenched linker and released drug.

**Plasma conjugate stability**

Quaternary ammonium linker conjugate stability was assessed in mouse and rat plasma. ADCs were prepared with 8 drugs per antibody with glucQ-AE 6 using fully reduced cAC10. The conjugate (1.0 mg/ml) was incubated in sterile rat and mouse plasma for 10 days at 37 °C. At 8 time points during the incubation, 50 µl aliquots of each ADC were removed and frozen at -80 °C. Upon completion of the time course, ADCs were purified from each sample by capture with IgSelect resin (GE Healthcare, Chicago, IL), which selectively binds to the human Fc domain, and analyzed by reversed-phase HPLC in line with a QTof mass spectrometer to determine the drug-to-antibody ratio (DAR) as a function of time.

**In vitro cytotoxicity assays**
In vitro potency was assessed on multiple cancer cell lines: L540cy and L428 (Hodgkin lymphomas); Karpas299 (obtained from Dr. Abraham Karpas, University of Cambridge), DEL, and DEL-BVR (20) (anaplastic large cell lymphomas), 786-O (renal cell carcinoma), and Ramos (non-Hodgkin lymphoma) cell lines. L428 and DEL cell lines were obtained from DSMZ (Braunschweig, Germany). 786-O and Ramos cell lines were obtained from ATCC (Manassas, VA). The L540cy cell line was provided by Dr. Harald Stein (Institut fur Pathologie, University of Veinikum Benjamin Franklin, Berlin, Germany). All cell lines were authenticated by STR profiling at IDEXX Bioresearch (Columbia, MO) and cultured for no more than two months after resuscitation. Cells cultured in log-phase growth were seeded for 24 hours in 96-well plates containing 150 μl RPMI 1640 supplemented with 20% FBS. Serial dilutions of antibody-drug conjugates in cell culture media were prepared at 4x working concentrations, and 50 μl of each dilution was added to the 96-well plates. Following addition of test articles, cells were incubated with test articles for 4 days at 37 °C. After 96 hours, growth inhibition was assessed by CellTiter-Glo® (Promega, Madison, WI) and luminescence was measured on a plate reader. The EC₅₀ value, determined in triplicate, is defined here as the concentration that results in half maximal growth inhibition over the course of the titration curve.

Tubulin fluorescence polarization competition binding assay

Sheep brain tubulin (SBT) was obtained from Cytoskeleton (Denver, CO) and exact protein concentration was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Eight point serial dilutions of test compounds were performed in assay buffer (20mM PIPES, pH 6.9, 1 mM EGTA + 0.008% Tween 20) + 60 nM FITC-MMAF probe for competition representing a 2x assay concentration (highest amount 250 μM and dilutions occurring at 5x concentration). To initiate the assay, 15 μl of (2x) test compound dilution + 60 nM FITC-MMAF was combined with 15 μl (2x) 600 nM SBT in assay buffer in the wells of a 384-well plate for a final concentration of 30 nM FITC-MMAF, 300
nM SBT, and 8 test compound concentration points performed in triplicate. The plate was covered and
the binding competition was allowed to proceed for 1 hour at room temperature with gentle shaking.
Fluorescence polarization was measured on an Envision multilabel reader (Perkin Elmer, Waltham, MA)
using an installed FITC FP dual mirror. Measurements of polarization (milli-polarization units) are
defined as \((\text{mP}) = 1000 \times \frac{(S-G*P)}{(S+G*P)}\) where \(S\) and \(P\) represent the parallel and perpendicular
background subtracted fluorescence count rates following polarized excitation, and \(G\) (grating) is an
instrument dependent factor calculated from pure fluorophore solution. Binding data was analyzed
using Graphpad Prism software (La Jolla, CA). EC50 values for the given assay conditions were calculated
from a dose-response variable slope model given by the equation \([Y = \text{Bottom} + \frac{(\text{Top-}
\text{Bottom})}{(1+10^{((\text{LogEC50-X})^{*}\text{HillSlope})}}]\).

In vivo xenograft models

All experiments were conducted in concordance with the Institutional Animal Care and Use
Committee in a facility fully accredited by the Association for Assessment and Accreditation of
Laboratory Animal Care. Therapy experiments were conducted in an L540cy Hodgkin lymphoma
xenograft model. Tumor cells, as a suspension, were implanted sub-cutaneous in immune-compromised
SCID mice. Upon tumor engraftment, mice were randomized to study groups when the average tumor
volume reached about 100 mm³. The ADCs were dosed once via intraperitoneal injection. Tumor
volume as a function of time was determined using the formula \((L \times W^2)/2\). Animals were euthanized
when tumor volumes reached 1000 mm³. Mice showing durable regressions were terminated around
day 90 post implant.

ADC pharmacokinetic experiments

Pharmocokinetic (PK) experiments were performed using radiolabeled antibody or ADC. To a
solution of mAb or ADC in PBS supplemented with an additional 50 mM potassium phosphate (pH 8.0)
and 50 mM sodium chloride was added 55 μCi N10 succinimidyl propionate, [propionate-2,3-3H]-
(Moravek Biochemicals, Brea, CA, 80 Ci/mmole, 1 mCi/ml, 9:1 hexane:ethyl acetate solution) per mg of
antibody or ADC. The resulting solution was vortexed and left at room temperature for 2 hours,
centrifuged at 4,000g for 5 minutes, and the lower aqueous layer was removed and split into 30 kDa
MWCO Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA). Unconjugated radioactivity
was removed by 4 rounds of dilution and centrifugation at 4,000g. The resulting products were filtered
through sterile 0.22 μm Ultrafree-MC Centrifugal Filter Units (Millipore, Billerica, MA) and the final
antibody or ADC concentration was measured spectrophotometrically. The specific activity (μCi/mg) of
each product was determined by liquid scintillation counting.

The PK properties of the unconjugated antibody or ADC were examined in two experiments. In
each experiment, 1 mg of radiolabeled antibody or ADC per kg of animal weight was injected via the tail
vein. Each test article was dosed once in replicate animals. Blood was drawn into K2EDTA tubes via the
saphenous vein or by cardiac puncture for terminal bleeds at various time points. Plasma was isolated
by centrifugation for 10 minutes at 10,000g. A 10-20 μl sample of plasma from each time point was
added to 4 ml Ecoscint-A liquid scintillation cocktail (National Diagnostics) and the total radioactivity was
measured by liquid scintillation counting. The resulting disintegrations per minute values were
converted to μCi and the specific activity of the radiolabeled test articles was used to calculate the mAb
or ADC concentrations remaining in the plasma at each time point.

Results

Drug-linker design

The new β-glucuronidase-cleavable drug-linkers developed in this study consist of auristatin E
(1) and tubulysin (3) conjugated at their N-terminal tertiary amine groups, leading to quaternary
ammonium drug-linkers glucQ-AE (6) and glucQ-Tub (8), respectively (Figure 2). The glucuronide linker system (18) is a hydrophilic alternative to commonly used protease-cleavable linkers, such as valine-citrulline (7) (val-cit) and valine-alanine (21, 22), and exploits intracellular β-glucuronidase to initiate drug release (23). The hydrophilic nature of the glucuronic acid moiety confers improved linker physicochemical properties and decreased ADC aggregation with lipophilic payloads such as minor groove binders (24) and camptothecins (6), making it an ideal linker choice to conjugate a lipophilic tubulysin analogue like compound 3.

A self-stabilizing maleimide (mDPR) was utilized as the point of attachment for antibody conjugation. Unlike unsubstituted alkyl maleimides, the mDPR group has been shown to stabilize through thiosuccinimide hydrolysis post-antibody alkylation, preventing de-conjugation in vivo (25). The quaternary ammonium drug-linkers 6 and 8 (Figure 2) were prepared by alkylation of tertiary amines with a glucuronide benzyl bromide linker intermediate, forming the quaternary ammonium linkage. The analogous carbamate-based drug-linkers 7 and 9 were prepared using established linker chemistries (18, 19).

**Drug release and conjugate stability of glucQ-AE**

Prior to evaluation of the auristatin drug-linkers 6 and 7 as conjugates, drug release from the quaternary ammonium linker 6 was investigated. The drug-linkers were quenched at the maleimides with N-acetylcysteine and treated with β-glucuronidase at 0.5 and 1 mg/ml. In the presence of enzyme, glucQ-AE (6) quantitatively released free drug AE (1) with minimal attenuation of kinetics relative to the gluc-MMAE (7) carbamate control (Figure 3). For example, in the presence of 0.5 mg/ml β-glucuronidase glucQ-AE was 33% cleaved compared to 72% cleavage for gluc-MMAE after incubation for 1 hour. There was no evidence in the extracted ion or UV/Vis chromatograms for the para-hydroxy-
benzylammonium intermediate, indicating rapid [1,6]-fragmentation to release AE. In the absence of enzyme, both drug-linkers were unchanged and evolution of free drugs was not observed.

To assess the stability of glucQ-AE linker 6, DAR 8 αCD30 ADCs were incubated at 37 °C in mouse and rat plasma and tested for linker integrity at various time points over the course of 10 days. LC/MS analysis of the samples revealed the conjugate to be unchanged (Supplementary Figure S1) over this time period, indicating a high degree of linker stability in biological matrices. These results are comparable to what was previously reported for a carbamate-linked glucuronide-MMAE linker, which also displayed stability in rat plasma (18).

**Auristatin E free drug and ADC in vitro cytotoxicity**

The auristatin free drugs AE (1) and MMAE (2) and αCD30 conjugates bearing the glucQ-AE (6) and gluc-MMAE (7) linkers were evaluated for in vitro cytotoxic activities. As shown in Table 1, AE trended 2.5-7 fold more potent than MMAE on three of the five cell lines tested, with the largest difference observed in the MDR+ L428 Hodgkin lymphoma cell line. Comparable potencies were observed in Karpas299 and Ramos cell lines. As DAR 8 αCD30 conjugates, similar trends were observed, as shown in Table 2. In all five CD30-positive lymphoma lines, both conjugates were highly potent, with EC50s in the sub-nM range. As with the free drug cytotoxicity, the glucQ-AE conjugate was 1.5-4 fold more potent than the gluc-MMAE comparator. Both linkers formats provided ADCs that were immunologically specific, with EC50s on the CD30-negative Ramos cell line greater than 2-3 logs higher than the CD30-positive lymphoma cell lines.

**Quaternary ammonium-linked auristatin E in vivo xenograft activity**

To validate the quaternary ammonium linker system in vivo, αCD30 conjugates bearing glucQ-AE (6) and gluc-MMAE (7) were evaluated in the L540cy Hodgkin lymphoma xenograft model. Anti-CD30
ADCs were prepared at an average of 4-drugs/Ab, as conjugates bearing 8-drugs/Ab of a gluc-MMAE linker have been shown to possess sub-optimal pharmacokinetic properties (19). Mice bearing L540cy xenografts were administered a single dose of test article once the average tumor volume reached 100 mm³ on day 10 (Figure 4A). Treatment with 0.8 mg/kg αCD30 conjugate bearing glucQ-AE was curative, while a lower dose of 0.4 mg/kg provided only a delay in tumor outgrowth. In contrast, mice treated with 0.8 mg/kg αCD30 conjugate bearing gluc-MMAE experienced a transient tumor regression with subsequent outgrowth in 5/5 mice. A lower dose of 0.4 mg/kg of the carbamate control provided a brief tumor growth delay. In both cases, non-binding IgG controls were inactive, indicating that the results were immunologically specific.

**Tubulysin free drug cellular and biochemical potency**

Having established that the quaternary ammonium linker with auristatin E provided stable, active, and immunologically specific ADCs, we turned our attention to tubulysin analogue 3. As shown in Table 1, tubulysin 3 displays high potency on cancer cell lines, with EC50s in the sub-nanomolar range. However, unlike auristatin E, modification of tubulysin 3 to provide a conventional handle for carbamate-based conjugation resulted in a significant attenuation of free drug potency. Replacement of the N-terminal tertiary amine in Tub 3 with a secondary amine provided desmethyltubulysin (DMTub) 4, which was 40-380 fold less potent in 4/5 cancer cell lines (Table 1). A loss of potency was also observed for the desmethyl analogue 4 in a biochemical potency assay (Figure 5). Free drugs 1-4 were compared in a tubulin binding assay for ability to compete with FITC-labeled monomethylauristatin F, a molecular probe that binds tubulin with high affinity (unpublished results). Consistent with the cytotoxicity results, DMTub 4 competes for tubulin binding with 33-fold lower affinity relative to unmodified Tub 3. In contrast, replacement of the tertiary amine in AE (1) with a secondary amine in MMAE (2) resulted in only a 2-fold loss in relative tubulin affinity. Finally, conversion of the carboxylic acid in Tub 3 to the
hydrazide, creating TubNHNH₂ (5), resulted in a 5-20 fold drop in free drug potency compared to the unmodified parent Tub 3 (Table 1). Thus, given its high free drug potency and the attenuated activity of N- and C-terminal analogues, Tub 3 is an ideal cytotoxic agent for antibody conjugation via the quaternary ammonium linker.

Quaternary ammonium-linked tubulysin ADC cytotoxicity

Glucuronide drug linkers bearing Tub 3 and DMTub 4 were synthesized as described to provide glucQ-Tub linker 8 and gluc-DMTub linker 9. Anti-CD30 ADCs were conjugated at 8-drugs/Ab and tested on a panel of CD30-positive lymphoma cell lines. As shown in Table 2, αCD30 ADC bearing the glucQ-Tub linker 8 was highly potent on all of the CD30-positive lymphoma cell lines. In contrast, αCD30-9, bearing gluc-DMTub, displayed variable potencies across the cell line panel and in all cases was impaired relative to the glucQ-Tub conjugate. For example, αCD30-9 was 30- and 100-fold less potent on MDR+ DEL-BVR and L428 cell lines, respectively. In contrast, the loss of potency was more modest (2.5-11 fold relative to the glucQ-Tub conjugate) on the MDR-negative L540cy, Karpas299, and DEL cell lines. Both linkers provided immunologically specific conjugates, with EC50s greater than 100 nM, the highest dose tested, on CD30-negative Ramos cells.

Quaternary ammonium-linked tubulysin in vivo xenograft activity

Anti-CD30 ADCs bearing glucQ-Tub 8 were evaluated in the L540cy HL xenograft model, as shown in Figure 4B and 4C. For the in vivo experiments, ADCs were conjugated at 4-drugs/Ab as higher loading at 8-drugs/Ab was found to cause accelerated clearance from circulation in rats (Supplementary Figure S2B). The αCD30 DAR 4 conjugate was initially evaluated at a single dose of 0.6 mg/kg in mice that were treated once the average tumor volume reached 100 mm³ on day 7 (Figure 4B). The αCD30-8 Tub conjugate proved highly potent, with cures in 5/5 mice. A subsequent L540cy xenograft was performed to test the conjugate at lower doses. Tumor volumes for each of the mice are shown in
Figure 4C. A dose of 0.15 mg/kg provided a brief tumor growth delay, while a higher dose of 0.3 mg/kg was more active, with most mice achieving transient tumor regressions and one cure.

Discussion

Carbamate-based linkage chemistry for antibody conjugation of primary (6, 7) and secondary (8) amines is well-established, leading to approaches where the tertiary amine-containing drugs serve as templates for the design of their secondary amine analogues. However, if the tertiary amine is part of a structural element or necessary for activity, such substitutions are counter-productive. Because of this, there is a need for conjugation technologies that utilize the tertiary amine element present within many cytotoxic drug payloads under consideration as ADC warheads. Precedence for the approach described here includes a prodrug strategy involving a quaternary ammonium salt of mechlorethamine (26), which liberated free drug under bioreductive conditions. Quaternary ammonium glucuronide prodrugs of verapamil and quinine have been reported to release free drugs in the presence of β-glucuronidase, albeit at 50-500 fold higher enzyme levels than required for carbamate-based analogues (27). While this work demonstrated stability in pH 7.2 PBS for 1 hour, it was unclear from these examples if a quaternary ammonium-based linker would be stable under physiologic conditions over the course of 1-2 weeks, the time scale commensurate with ADC circulation half-lives. In addition, the efficiency of drug release by lysosomal β-glucuronidase was not known. These points were resolved in the studies described here, as evidenced from the drug stability characteristics, and the high potencies and specificities of the ADCs containing quaternary ammonium linkers. Recently, in parallel with our ADC efforts, a val-cit-dipeptide based quaternary ammonium linker was reported for the conjugation of a rifalogue antibiotic for an antibody-antibiotic conjugate (AAC) approach to treat intracellular methicillin-resistant S. aureus (28). Taken together, these results underscore the applicability of this approach for targeted drug delivery.
Replacement of the tertiary amine in auristatin E for a secondary amine in monomethylauristatin E is a tolerated drug modification, providing the basis to evaluate the quaternary ammonium linker strategy relative to a comparable carbamate control. The quaternary ammonium glucuronide-AE linker 6 possessed the hallmarks of an effective ADC linker – rapid drug release (Figure 3), extended conjugate stability (Supplementary Figure S1), and potent and immunologically specific ADCs. The increased in vivo activity (Figure 4A) of αCD30-6 over the carbamate control αCD30-7 may be attributed to increased free drug potency and increased PK exposure of the higher loaded ADCs bearing the quaternary ammonium linker. The DAR 4 αCD30 conjugates evaluated in vivo are a Gaussian distribution of species ranging from 0 to 8 drugs/mAb. The quaternary ammonium linker glucQ-AE (6) provided DAR 8 ADCs with greater PK exposure (Supplementary Figure S2A) than the gluc-MMAE (7) comparator, possibly due to increased hydrophilicity conferred by the permanent positive charge on the quaternary ammonium group.

The tubulysins are a compelling drug class for ADCs as they are highly potent across a wide range of cancer cell lines and retain potency on MDR+ cell lines like L428 and 786-O (Table 1). We and others (15) have demonstrated that a secondary amine at the N-terminus of two distinct tubulysin analogues significantly impairs cytotoxic activity relative to the N-methylated tertiary amine parent compounds. Conversion of the C-terminal carboxylate to include a circulation-stable conjugatable group appears more promising. For example, a modest 3-fold reduction in free drug potency on KB cells was observed upon conversion of the carboxylate in tubulysin B to the corresponding hydrazide as part of a folate-targeted conjugate (16). However, with tubulysin 3, conversion to the hydrazide (5) resulted in around a 5-20 fold loss of potency (Table 1). Another logical conjugation approach is the incorporation of an aryl amine in the tubuphenylalanine side chain, enabling direct peptide conjugation. Indeed, introduction of an aniline functional group in the para position of the phenyl ring has been reported for several tubulysin analogues and appears to consistently attenuate potency (17). Thus, given the
sensitivity of tubulysins toward modification, it is preferable to utilize conjugation strategies that permit release of the chemically unmodified forms of the drug.

The quaternary ammonium linker is a new and viable strategy for arming antibodies with tertiary amine-containing payloads, providing circulation-stable, potent and immunologically specific ADCs. The technology should apply to an array of drugs, and we continue to expand the work to include new tertiary amine containing compounds. In addition, future work will focus on further evaluating the activities of the promising ADCs described as potential development candidates.

References

Tables

**Table 1.** EC50 values† (nM drug) for free drugs following a 96 h incubation

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<th>compound</th>
<th>drug</th>
<th>L540cy, HL</th>
<th>L428ǂ, HL</th>
<th>Karpas299, ALCL</th>
<th>786-Oǂ, RCC</th>
<th>Ramos, NHL</th>
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†Following treatment, cells were assessed for viability as described in the Materials and Methods.

ǂMDR-positive by rhodamine efflux

**Table 2.** EC50 values† (nM drug) for αCD30 glucuronide-based DAR 8 ADCs

| ADC cytotoxicity | αCD30 lymphoma cell lines | Ramos (CD30-)
<table>
<thead>
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<td>L428ǂ, HL</td>
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†Cells were treated for 96 hours, then assessed for cell viability as described in the Materials and Methods.

ǂMDR-positive by rhodamine efflux

**Figure Legends**

**Figure 1.** Structures of free drugs (Figure 1A) and glucuronide linker drug release mechanism (Figure 1B)

**Figure 2.** Structures of auristatin and tubulysin glucuronide drug-linkers
Figure 3 - Kinetics of drug release in the presence of β-glucuronidase. Linker maleimides were quenched with N-acetylcysteine (NAC) prior to enzymatic digestion. Both glucuronide quaternary ammonium (6) and carbamate (7) linkers are quantitatively cleaved, rapidly evolving free drugs AE (1) and MMAE (2), respectively. β-glucuronidase concentrations are indicated in each case.

Figure 4 – Antitumor activities of quaternary ammonium-linked auristatin and tubulysin conjugates. Mice with CD30+ L540cy Hodgkin lymphoma xenografts were treated with a single i.p. dose of test article once the average tumor volume reached 100 mm³, as indicated with an arrow. (A) Quaternary ammonium-linked AE (6) was more potent than the carbamate-linked MMAE control (7) (n=5 mice/group). Plotting was discontinued once tumor volume exceeded 1000 mm³ for at least one animal. (B) Quaternary ammonium-linked Tub (8) was curative in the CD30+ L540cy model following dose of 0.6 mg/kg (n=5 mice/group). (C) Quaternary ammonium-linked Tub (8) was evaluated at lower doses of 0.15 and 0.3 mg/kg (n=6 mice/group).

Figure 5 – Relative tubulin binding affinity of auristatin and tubulysin free drugs in a tubulin fluorescence polarization assay. Replacement of the tertiary amine in tubulysin 3 with a secondary amine, creating DMTub 4, results in a 33-fold loss in binding affinity.
Figure 1.

A. Structures of auristatin and tubulysin analogs

1. R=Me, auristatin E (AE)
2. R=H, monomethylauristatin E (MMAE)
3. R'=Me, R''=OH, tubulysin analogue (Tub)
4. R'=H, R''=OH, desmethyltubulysin (DMTub)
5. R'=Me, R''=NHNH₂, tubulysin hydrazone (TubNHNH₂)

B. Mechanism of β-glucuronidase mediated drug release from the glucuronide drug-linker systems
Figure 3.

**Normalized linker ion counts,**
**LC/MS assay**

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**Free drug ion counts,**
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Figure 4.
Figure 5.

Tubulin binding fluorescence polarization assay

Log Inhibitor [M]

mP

AE, 1
MMAE, 2
Tub, 3
DMTub, 4

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