Optimization of a PEGylated glucuronide-monomethylauristatin E linker for antibody-drug conjugates


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Abstract

The emergence of antibody-drug conjugates (ADCs) such as brentuximab vedotin and ado-trastuzumab emtansine has led to increased efforts to identify new payloads and develop improved drug-linker technologies. Most antibody payloads impart significant hydrophobicity to the ADC, resulting in accelerated plasma clearance and suboptimal in vivo activity, particularly for conjugates with high drug-to-antibody ratios (DAR). We recently reported on the incorporation of a discrete PEG24 polymer as a side chain in a β-glucuronidase-cleavable monomethylauristatin E (MMAE) linker to provide homogeneous DAR 8 conjugates with decreased plasma clearance and increased antitumor activity in xenograft models relative to a non-PEGylated control. In this work, we optimized the drug-linker by minimizing the size of the PEG side chain and incorporating a self-stabilizing maleimide to prevent payload de-conjugation in vivo. Multiple PEG-glucuronide-MMAE linkers were prepared with PEG size up to 24 ethylene oxide units, and homogeneous DAR 8 ADCs were evaluated. A clear relationship was observed between PEG length and conjugate pharmacology when tested in vivo. Longer PEG chains resulted in slower clearance, with a threshold length of PEG8 beyond which clearance was not impacted. Conjugates bearing PEG of sufficient length to minimize plasma clearance provided a wider therapeutic window relative to faster clearing conjugates bearing shorter PEGs. A lead PEGylated glucuronide-MMAE linker was identified incorporating a self-stabilizing maleimide and a PEG12 side chain emerged from these efforts, enabling highly potent, homogeneous DAR 8 conjugates and is under consideration for future ADC programs.

Introduction

Antibody-drug conjugates (ADCs) seek to combine the exquisite specificity of an antibody to its cognate antigen with the cytotoxic potency of the drug payload. Advances in ADC technology have
paved the way for two FDA-approved ADCs: CD30-targeted brentuximab vedotin for relapsed and refractory Hodgkin lymphoma and ALCL (1), and HER2-targeted ado-trastuzumab emtansine for HER2-expressing breast cancer (2). There are many design factors crucial to the development of effective ADCs (3-5), and plasma pharmacokinetics (PK) has emerged as an important parameter on ADC pharmacology (6).

Payload conjugation to antibodies can impart significant hydrophobicity to the resulting conjugate, which can lead to ADC aggregation and/or accelerated plasma clearance in vivo, often limiting the drug-to-antibody ratio. As a consequence, the majority of ADCs in clinical development are loaded with an average of only 2-4 drugs per antibody (7). This limitation is due not only to the intrinsically hydrophobic character of the cytotoxic agent employed as the drug payload, such as the antimitotic monomethylauristatin E (MMAE) in Figure 1, but also to the linker technology employed to ensure the release of the drug in a chemically unmodified form. For example, self-immolative cleavable linker systems like the valine-citrulline (val-cit) dipeptide (drug-linker 1) and glucuronides (drug-linkers 2-10) rely on a [1,6]-fragmentation across a hydrophobic aryl spacer to achieve drug release, adding additional hydrophobicity to the conjugate. Recently, we reported that a DAR 8 ADC prepared with the hydrophobic drug-linker val-cit-monomethylauristatin F was taken up by Kupffer cells and hepatic sinusoidal endothelial cells, likely contributing to the accelerated plasma clearance in rats (6). This uptake was greatly diminished when using drug-linkers designed to minimize hydrophobicity, and the resulting ADCs exhibited plasma pharmacokinetics similar to that of the unconjugated antibody.

While an effective drug-linker for ADCs (8), drug-linker 1 (Figure 1) has limitations. The physicochemical properties of the drug-linker limit the optimal drug-loading to approximately 4 drugs per antibody when conjugated as a distribution across four reducible native antibody cysteine disulfides. Early work with drug-linker 1 demonstrated that higher loaded DAR 8 conjugates, while more potent...
than lower loaded ADCs in vitro, were inferior in vivo due to accelerated plasma clearance (9). In this work, we sought to achieve greater ADC potency and homogeneous DAR 8 conjugates by stably conjugating MMAE via a hydrophilic PEGylated glucuronide linker system.

The glucuronide linker system represents a hydrophilic alternative to the val-cit dipeptide, and exploits lysosomal β-glucuronidase (10) to initiate drug release. The glucuronide linker has been used to mitigate aggregation issues with lipophilic payloads like minor groove binders (11) and camptothecins (12). Glucuronide-MMAE linker 2 was found to provide ADCs at 4-drugs/Ab with in vitro and in vivo properties similar to val-cit-MMAE linker 1 conjugates (13). More recently, we have shown that incorporation of PEG24 solubilizing moiety in a side chain configuration (drug-linker 3), but not as a tether between the maleimide and drug, enabled ADCs loaded at 8-drugs/Ab with greater plasma exposure and improved in vivo performance relative to non-PEGylated drug-linker 2 controls (6).

A second limitation of drug-linker 1 is the utilization of the maleimidocaproyl (MC) stretcher unit for covalent attachment of the drug-linker to the targeting antibody. Simple alkyl maleimides are known (14) to undergo partial de-conjugation in vivo, as the reaction of the Cys thiol with the maleimide is reversible and in vivo the drug-linker maleimide can be captured by endogenous albumin (14) (Figure 1). Once the thiosuccinimide product of the maleimide conjugation undergoes ring hydrolysis, however, the covalent linkage of the drug-linker to the antibody is stabilized. The self-stabilizing maleimide (mDPR, Figure 1) was developed to undergo rapid post-conjugation thiosuccinimide hydrolysis, to provide circulation-stable ADCs with a wider therapeutic window (15).

The goal of this work was to optimize conjugate delivery of MMAE by combining these advancements to maximize ADC potency and therapeutic window from homogeneous DAR 8 conjugates. Specifically, we sought to optimize drug-linker 3 through incorporation the mDPR self-stabilizing maleimide and investigate the effects of PEG length on pharmacological properties.
Materials and Methods

Preparation of ADCs bearing mDPR-PEG₃-glucuronide-MMAE linkers

A detailed experimental procedure for the preparation of the drug-linkers has been previously described (6, 16). Anti-CD30 and anti-CD19 antibodies, cAC10 and hBU12, respectively, were conjugated as previously described (17). Briefly, a solution of antibody around 10 mg/ml in PBS (pH 7.4) containing 1 mM EDTA was treated with 12 equivalents of tris(2-carboxyethyl)-phosphine (TCEP) to achieve full reduction of the four interchain disulfides. Reduction progress was monitored by reversed-phase chromatography and additional TCEP was added as needed to complete the reaction. Upon reaction completion, the reduced antibody solution was purified by three rounds of ten-fold dilution and centrifugation at 4,000 g through a 30 kDa molecular weight cutoff (MWCO) filter.

Fully reduced antibody as a PBS solution containing 1 mM EDTA and buffered with 100 mM potassium phosphate to pH 7.4 was 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 eight available mAb cysteines were alkylated, the crude ADC solution was purified by three rounds of ten-fold dilution with PBS and centrifugation at 4,000 g through a 30 kDa MWCO filter. The extent of aggregation was assessed by size exclusion chromatography (SEC), and in all cases, monomeric ADCs were obtained with less than 2% aggregation. The SEC chromatograms revealed a shift to shorter retention times as the PEG length in the drug-linker increased, indicative of an increase in apparent ADC size (Supplemental Figure S1). The resulting DAR 8 homogeneous ADCs were sterile-filtered through a 0.22 µm centrifugal filter and stored at -80 °C.

In vitro cytotoxicity assays
In vitro potency was assessed on multiple cancer cell lines: L540cy and L428 (Hodgkin lymphomas); Karpas299 (anaplastic large cell lymphoma, originally provided by Dr. Abraham Karpas, University of Cambridge), Ramos (Burkitt’s lymphoma), and SU-DHL-4, WSU-DLCL2, and RL (diffuse large B-cell lymphoma) cell lines. L428, Karpas299, SU-DHL-4, and WSU-DLCL2 cell lines were obtained from DSMZ (Braunschweig, Germany). Ramos and RL 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). The CD30+ Karpas299, L540cy, and L428 lymphoma cell lines were obtained in 2012. The CD19+ Ramos, SU-DHL-4, WSU-DLCL2, and RL lymphoma cell lines were obtained in 2013. 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 ADC, cells were incubated with test articles for 4 days at 37 °C, after which growth inhibition was assessed by CellTiter-Glo® (Promega, Madison, WI). The EC50 value, determined in triplicate, is defined as the concentration that achieves half-maximal growth inhibition over the course of the titration curve.

In vivo tolerability and therapy experiments

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. To assess mouse tolerability, Balb/C mice (n=3) were treated with a single intraperitoneal dose of 50 mg/kg of ADC on day 0. The mice were then observed for weight loss or outward signs of toxicity over the course of nine days. Mice experiencing greater than 20% weight loss were euthanized.
Efficacy experiments were conducted in CD19-expressing RL and WSU-DLCL2 models of diffuse large B-cell lymphoma (DLBCL) and the Raji model of Burkitt’s lymphoma. Tumor cells, as a suspension, were implanted sub-cutaneously in immune-compromised SCID mice. Upon tumor engraftment, mice were randomized to study groups when the average tumor volume reached about 100 mm³. Test articles were administered once via intraperitoneal bolus 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.

**Intratumoral concentration of MMAE**

Subcutaneous L540cy xenografts were grown to 200 mm³ prior to dosing with the indicated cAC10 ADCs at 3 mg/kg. After 3 days, tumors were harvested and weighed, and MMAE concentrations within the tumor homogenate were determined by LC-MS/MS. Deuterium-labeled MMAE (d8) as an internal standard was spiked into the tumor sample, followed by 1 ml of methanol. A known quantity of the tumor was homogenized with a Tissue Tearor (Biospec Products, Bartlesville, OK) and centrifuged. The supernatant was transferred to a 96 deep well plate and dried under a stream of nitrogen. The residual was reconstituted in 20% methanol, 0.1% formic acid, and analyzed by LC-MS/MS on a Kinetix C18 reversed phase column (Phenomenex) coupled to a Waters Quattro triple quadrupole mass spectrometer. Quantitation was performed using standards prepared from untreated tumors that were spiked with MMAE and processed in a manner identical to the samples. The density of the tumor tissue was taken to be 1.0 which was used to calculate the nanomolar concentration of MMAE within the tumor tissue.

**ADC pharmacokinetic (PK) experiments**

Pharmacokinetic experiments were performed using radiolabeled antibodies and ADCs. To a solution of antibody 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/mmol, 1 mCi/ml, 9:1 hexane:ethyl acetate solution) per mg of
antibody or ADC. The resulting mixture was vortexed and left at room temperature for 2 hours. The
mixture was centrifuged at 4,000g for 5 minutes and the lower aqueous layer was removed and split
into Amicon 30 kDa MWCO 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 pharmacokinetic properties of the unconjugated antibody or ADC were examined in
Sprague-Dawley rats. In each experiment, 3 mg of radiolabeled antibody or ADC per kg of animal weight
were injected via the tail vein. Each test article was dosed once in three replicate animals. Blood was
drawn into K2EDTA tubes via the saphenous vein at various time points or by cardiac puncture for
terminal bleeds. Plasma was isolated by centrifugation for 10 minutes at 10,000g. A 10-20 μl of sample
of plasma from each time point was added to 4 ml Ecoscint-A liquid scintillation cocktail (National
Diagnostics, Atlanta, GA) 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 concentration of antibody or ADC remaining in the
plasma at each time point. The data were fit to a two-compartment model to derive the clearance rates
using WinNonLin.

Results

Synthesis of drug-linkers
The drug-linkers were synthesized (6, 16) from the known intermediate glucuronide-MMAE (Figure 2). Fmoc-L-Lys-OH was acylated at the ε-amine with N-hydroxysuccinimide (NHS)-activated methoxy-PEGₙ reagents (or Ac₂O for drug-linker 9) to provide Fmoc-Lys(PEGₓ)-OH intermediates. The free acid was then converted to the activated ester, and subsequently reacted with glucuronide-MMAE. The lysine Nα-Fmoc was then removed under standard conditions and reacted with mDPR(Boc)-OSu to provide the penultimate intermediates. Finally, the Boc group was removed to provide mDPR-{PEGₓ}-glucuronide-MMAE drug-linkers 4-9. Drug-linker controls included an Nε-acetylated construct to assess the role of the lysine residue (drug-linker 9) and a drug-linker (10) lacking the lysine group altogether. For drug-linker 10, glucuronide-MMAE was reacted directly with mDPR(Boc)-OSu to provide mDPR(Boc)-glucuronide-MMAE, which upon Boc-deprotection gave the desired product.

**mDPR inclusion increases MMAE delivery in vivo and stability**

It has previously been reported that replacement of the MC stretcher with mDPR has no effect on in vitro ADC potency and activity, but that the improved stability may lead to increased in vivo potency in a model-dependent manner (15). Based on these results, the impact of mDPR inclusion on intratumoral drug delivery was assessed. Mice bearing subcutaneous 200 mm³ CD30⁺ L540cy xenografts were administered a single i.p. dose of 3 mg/kg of DAR 8 αCD30 ADCs bearing drug-linkers 2-4, the tumors were harvested 3 days later, and the intratumoral drug concentration was assessed by mass spectrometry (Figure 3). The group treated with αCD30-2 had a lower intratumoral drug concentration of MMAE compared to αCD30-3, consistent with the accelerated clearance of DAR 8 ADCs comprised of drug-linker 2 (6). Lastly, a modest upward trend in drug delivery over αCD30-3 was observed with the incorporation of the self-stabilizing maleimide, as shown in αCD30-4. As observed previously for drug-linkers containing mDPR (15, 18), ADC bearing drug-linker 4 was highly stable over...
the course of 7 days at 37 °C in rat plasma; whereas, ADCs comprised of MC-containing drug-linkers 2 and 3 underwent extensive de-conjugation under the same conditions (Supplemental Figure S2).

**In vitro cytotoxicity**

Anti-CD30 and αCD19 DAR 8 conjugates were tested on a panel of CD30+ and CD19+ cell lines. The cells were treated continuously for 96 hours and then assessed for viability. All of the drug-linkers tested provided potent and immunologically specific ADCs. On the CD30+ lymphoma lines, PEG inclusion had no effect on conjugate potency, with comparable EC50s observed across the cell line panel (Table 1). All αCD30 ADCs were inactive on CD30-negative Ramos cells, indicating a high degree of immunological specificity. The dose-response curves indicate that PEG did not impact maximal inhibition (Supplemental Figure S3). On the CD19+ Ramos and SU-DHL-4 lymphoma cell lines the presence of PEG had minimal effect on EC50 potency (Table 2); whereas, some variability in EC50 potency was observed in CD19+ WSU-DLCL2 and RL cell lines. On the RL, WSU-DLCL-2, and SU-DHL-4 DLBCL cell lines the presence of larger PEGs had an effect on maximal activity. Unlike on Ramos Burkitt’s lymphoma cell line and the CD30+ cell lines, the dose-response curves (Supplemental Figure S3) revealed an attenuation in maximal cell inhibition on the DLBCL lines for the PEGylated conjugates. It is unclear why this is the case, but may be attributable to some feature of the underlying biology of the DLBCL lines that produces this result.

**ADC pharmacokinetic properties and tolerability**

The effects of PEG size on ADC pharmacokinetic properties were determined using drug-linkers 4-10 attached to a non-binding IgG with a DAR of 8. Sprague-Dawley rats were administered a single i.v. dose of 3 mg/kg, and circulating antibody was monitored over time (Figure 4A). ADC exposure increased as a function of PEG size up to PEG8, at which point further increasing PEG size had little impact on exposure. Conjugates bearing PEG8 or larger PEGs displayed PK properties approaching those of the
parental antibody. The PK data were fit to a 2-compartment model to generate pharmacokinetic parameters. Clearance as a function of PEG size is plotted in Figure 4B. Clearance rapidly increased for all PEG conjugates bearing PEGs smaller than PEG8 or lacking PEG entirely (drug-linkers 9 and 10).

To evaluate the effect of ADC clearance on tolerability, Balb/C mice were administered a single dose of 50 mg/kg i.p. of DAR 8 humanized IgG1 conjugates bearing the PEGx series on day 0 and monitored for weight loss as a function of time. As shown in Figure 4C, ADCs bearing PEG sufficient in size to optimize PK exposure (PEG8, PEG12, and PEG24) were well tolerated with minimal, transient weight loss observed over the course of 9 days. In contrast, ADCs bearing PEGs smaller than PEG8 (drug-linkers 7 and 8) or lacking PEG altogether (drug-linkers 9 and 10) were not tolerated, with some members in each of the four groups euthanized with greater than 20% weight loss. As shown in Figure 4D, the surviving fraction increased as PK exposure increased, with 100% survival for ADCs bearing PEGs sufficient to preserve exposure (PEG8, PEG12, and PEG24).

After observing these tolerability differences in mice, two ADCs were selected representing both ends of the tolerability spectrum to evaluate in a more detailed study. Conjugates bearing PEG12 (drug-linker 5) or lacking PEG altogether (drug-linker 10) were again dosed at 50 mg/kg in Balb/C mice, which were sacrificed 3 days later for necropsy. Histology of the bone marrow revealed that the faster-clearing ADC prepared with drug-linker 10 resulted in substantially greater hypocellularity and hemorrhage compared to the slower clearing ADC bearing drug-linker 5 (Supplemental Figure S4). This elevated myelosuppression was recapitulated in the hematology results, with white blood cell counts in particular being more severely depressed in animals receiving the faster-clearing ADC (Supplemental Figure S5). Two of the three animals receiving this ADC also exhibited pronounced neutropenia. Collectively, these results suggest that bone marrow is the dose-limiting target organ of these ADCs, consistent with results published for other MMAE-containing drug-linkers (15).
In vivo xenograft models

A subset of the PEGylated drug-linkers were evaluated in a xenograft model to assess the effect of PEG size and PK properties on in vivo activity. DAR 8 αCD19 conjugates bearing PEG24, PEG12, PEG8, PEG4, and no PEG (drug-linkers 4, 5, 6, 7, and 10, respectively) were administered to mice bearing CD19+ RL diffuse large B-cell lymphoma xenografts. A single i.p. dose at 3 mg/kg was given once the average tumor volume reached 100 mm³. ADCs bearing PEG side chains sufficient in size to preserve parental antibody PK properties (PEG8, PEG12, and PEG24) led to much more pronounced activity than faster clearing conjugates without PEG (drug-linker 10) or with the PEG4 (drug-linker 7)-containing ADC. For example, αCD19-10 displayed the most rapid clearance in the PK experiment and had minimal activity, while almost all of the animals treated with PEG8, PEG12, and PEG24-containing ADCs experienced cures or complete regressions of their tumors. Thus, as in the tolerability experiment, in vivo activity correlated with conjugate PK properties, with the highest activity observed for ADCs bearing PEGs sufficient to maximize exposure.

The mDPR-(PEG12)-glucuronide-MMAE conjugate αCD19-5 was further evaluated in comparison to MC-val-cit-MMAE (drug-linker 1). Previous work has established that DAR 8 αCD30-1 val-cit-MMAE conjugates exhibit accelerated plasma clearance, providing inferior in vivo activity compared to longer circulating DAR 4 conjugates (9). Therefore, the αCD19-1 conjugate was prepared at 4-drugs/Ab, its optimal level of drug loading. Mice bearing CD19+ Raji (Burkitt’s lymphoma) xenografts were administered a single i.p. dose of conjugate on day 8 when the average tumor volume reached 100 mm³. As shown in Figure 5B, the DAR 8 αCD19-5 PEG12-glucuronide-MMAE conjugate dosed at 3 mg/kg achieved 10/10 cures, while the DAR 4 αCD19-1 val-cit-MMAE conjugate induced only modest tumor growth delay at the same mAb dose. At a lower dose of 1 mg/kg the αCD19-5 PEG12-glucuronide-MMAE conjugate provided antitumor activity similar to the val-cit-MMAE conjugate at 3 mg/kg. Thus, the
PEG₁₂-glucuronide-MMAE linker 5 provided ADC with comparable antitumor activity to those bearing val-cit-MMAE linker 1 at 1/3 of the antibody dose. Both drug-linkers provided inactive non-binding conjugates (IgG-1 and IgG-5) at the highest dose tested, indicating a high degree of immunological specificity.

The conjugates were also evaluated in mice bearing CD19+ WSU-DLCL2 (diffuse large B-cell lymphoma) xenografts. Mice were administered a single i.p. dose on day 11 when the average tumor volume reached 100 mm³. The DAR 8 αCD19-5 PEG₁₂-glucuronide-MMAE conjugate dosed at 1 mg/kg and 3 mg/kg induced 7/10 and 10/10 transient, complete regressions, respectively (Figure 5C). In comparison, the DAR 4 αCD19-1 (val-cit-MMAE-containing conjugate) dosed at 3 mg/kg achieved 5/10 transient, complete regressions. As shown before in the Raji model, the DAR 8 PEG₁₂-gluc-MMAE conjugate displayed superior activity compared to the DAR 4 val-cit-MMAE conjugate at 1/3 the antibody dose. In both cases, non-binding IgG controls had no effect on tumor outgrowth.

Discussion

Recently, we have shown that incorporation of a PEG₃₄ moiety configured in a side-chain orientation (converting drug-linker 2 to drug-linker 3) resulted in DAR 8 conjugates with diminished non-specific plasma clearance and consequently greater ADC exposure (6). We sought to improve upon this drug-linker through stabilization of the maleimide linkage and optimization of the size of the PEG side chain. For the purposes of maleimide stabilization, the MC-stretcher in drug-linker 3 was replaced with a self-stabilizing mDPR (drug-linker 4), leading to a small enhancement in intratumoral drug delivery (Figure 3). Although MC- versions of the entire PEG₉ series were not evaluated, previous findings would indicate that the optimized conjugate benefits from both increased stability of the maleimide linkage (15) and increased ADC exposure by PEGylation (6). To optimize the PEG size required for the glucuronide-MMAE linker combination, a series of PEGylated constructs was synthesized, conjugated at
8 drugs/Ab, and evaluated in vitro and in vivo. On most of the lymphoma cell lines, PEG had no effect on conjugate potency. On SU-DHL-4, WSU-DLCL2, and RL DLBCL cells PEG did appear to attenuate maximal inhibition (Supplemental Figure S3). Interestingly, the difference in maximum cell kill in vitro on RL cells didn’t appear to translate in vivo. In the RL xenograft model (Figure 5A), the activity trends correlated with plasma exposure, not attenuated in vitro activity. It seems reasonable to surmise that pharmacokinetic effects dominate small differences in in vitro activity in this case.

In vivo, a clear relationship was observed between PEG size and plasma pharmacokinetics, and ultimately the therapeutic window. ADC clearance in rats decreased as PEG unit length increased up to a threshold of PEG$_8$, at which point larger PEGs had little impact and ADC pharmacokinetics approximated that of parental antibody (Figure 4A). This result suggests that PEG$_8$ is sufficient to shield the hydrophobic moieties of the drug-linker and consequently minimize the non-specific cellular uptake of highly loaded ADCs that we have previously documented (6). This minimization of non-specific ADC clearance resulted in improved tolerability in a rodent model (Figure 4C). Since ADC clearance results in the catabolic release of active drug, we postulate that the faster-clearing ADCs produce higher concentrations of drug in the tissues where non-specific clearance is occurring, with diminished tolerability as a consequence. Conversely, results from a xenograft model (Figure 5A) indicated greater activity was obtained with ADCs bearing PEG units sufficient in length to maximize plasma exposure. Thus, the therapeutic window appeared to benefit from both reduced toxicity and greater activity when ADC pharmacokinetics were optimized by inclusion of an appropriately sized and configured PEG chain. It is noteworthy that toxicity appears to be inversely correlated with ADC exposure whereas antitumor activity is directly correlated with the same parameter. We believe that this is consistent with the underlying principles of antibody-based drug delivery – that antigen-mediated uptake at the tumor is optimized by maximizing exposure to the conjugate, while off-target toxicity is diminished by minimizing antigen-independent ADC uptake and clearance.
Within the context of the glucuronide-MMAE linker system, PEG₈, PEG₁₂, and PEG₂₄ all appeared to have the same ADC therapeutic window in rodents. Drug-linker 5, mDPR-(PEG₁₂)-glucuronide-MMAE, may be optimal for further development since it reduces by half the size of the conjugated PEG chain relative to the PEG₂₄ analog, which should lead to decreased overall hydrodynamic volume of the resulting ADC (Supplemental Figure S1). PEG₁₂ (drug-linker 5) was prioritized over PEG₈ (drug-linker 6) due largely to the clearance profiles shown in Figure 4B. As PEG length decreased from PEG₈ to PEG₄ the clearance doubled from 9.0 to 18.8 mL/kg·day. In rats it appears that ADCs loaded with PEG₈-glucuronide-MMAE are perilously close to the threshold for accelerated plasma clearance. It is unknown whether the threshold for accelerated clearance would reside between PEG₄ and PEG₈ in higher species. Therefore, PEG₁₂ was deemed the best option to balance PEG length reduction and mitigate the risk of interspecies differences.

We have demonstrated with a PEG₄-glucuronide-MMAE linker series that homogeneous DAR 8 ADCs with optimal ADC exposure provide a wider therapeutic window than conjugates cleared more rapidly. Furthermore, these data reinforce the concept that drug loading beyond the conventional 2-4 drugs/Ab is feasible provided payload hydrophobicity is accommodated through drug-linker design. Drug-linker 5, mDPR-(PEG₁₂)-glucuronide-MMAE, enables conjugation of MMAE to provide highly potent, homogeneous DAR 8 ADCs with a promising preclinical profile and is the subject of further investigation.

References


### Tables

#### Table 1. EC_{50} values† (ng ADC/ml) for αCD30 conjugates bearing PEG_{x}-glucuronide-MMAE linkers

<table>
<thead>
<tr>
<th>ADC</th>
<th>PEG_{x}</th>
<th>Karpas299, ALC</th>
<th>L540cy, HL</th>
<th>L428, HL</th>
<th>Ramos, NHL (CD30-)</th>
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†EC_{50} is defined here as the concentration that achieves half-maximal growth inhibition over the titration curve.

#### Table 2. EC_{50} values† (ng ADC/ml) for αCD19 conjugates bearing PEG_{x}-glucuronide-MMAE linkers

<table>
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<th>ADC</th>
<th>PEG_{x}</th>
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<th>SU-DHL-4, DLBCL</th>
<th>WSU-DLCL2, DLBCL</th>
<th>RL, DLBCL</th>
<th>L540cy, HL (CD19-)</th>
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<td>4</td>
<td>11</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

†EC_{50} is defined here as the concentration that achieves half-maximal growth inhibition over the titration curve.

### Figure Legends

**Figure 1.** Structures of monomethylauristatin E drug-linkers and self-stabilizing maleimide (mDPR)

**Figure 2.** General synthetic scheme to prepare mDPR-(PEG_{x})-gluc-MMAE linkers.

**Figure 3 - Effect of mDPR inclusion on intratumoral drug delivery.** Mice bearing CD30+ L540cy tumors were treated with 3 mg/kg αCD30 conjugates loaded at 8-drugs/mAb. Tumors were harvested 3 days post-dose and analyzed for intratumoral MMAE concentration.

**Figure 4 - ADC PK and tolerability as a function of PEG size.** (A) Conjugate exposure was assessed in Sprague-Dawley rats following a single i.v. dose at 3 mg/kg of ADCs bearing different size PEGs. (B) The PK data were fit to a two-compartment model to derive clearance rates as a function of PEG size. Clearance rates increase rapidly for conjugates bearing PEGs smaller than PEG_{B}. (C) Conjugate
tolerability was assessed in Balb/C mice (n=3) following a single i.p. dose of PEGylated conjugates at 50 mg/kg. Conjugates bearing PEGs smaller than PEG8 were not tolerated. Plotting was discontinued before 9 days when at least one cohort member was euthanized with greater than 20% weight loss. (D) Mouse tolerability plotted by survival as a function of time.

**Figure 5** – Antitumor activity of PEGylated conjugates in CD19+ lymphoma models following a single i.p. dose of test article once average tumor volume had reached 100 mm³. Plotting was discontinued once tumor volume exceeded 1000 mm³ for at least one cohort member. (A) A sub-set of PEGylated αCD19 conjugates were evaluated in the RL DLBCL model, dosing occurred on day 14. (B) and (C) The PEG12 drug-linker 5 was compared to the val-cit dipeptide drug-linker 1 in Raji and WSU-DLCL2 lymphoma models, respectively. Anti-CD19 conjugates were prepared at DARs corresponding to loadings that maximize ADC PK properties for each drug-linker. Dosing occurred on day 8 for the Raji model and day 11 for the WSU-DLCL2.
Figure 1

Disposed monomethylauristatin E (MMAE)

Drug-linker 1 - MC-val-cit-MMAE linker

Drug-linker 2 - n=0, MC-glucuronide-MMAE

Drug-linker 3 - n=1, MC-PEG24-glucuronide-MMAE

Ab-SH

mDPR

drug

Conjugation

Fast

Deconjugation

Thiosuccinimide hydrolysis

Fast

Note: step is slow with caproyl spacers

Stabilized linkage

Ab-SH
Figure 3

Drug-linker #  2  3  4
maleimide:    MC  MC  mDPR
PEG:          -  PEG24  PEG24
Figure 4

A. ADC rat pharmacokinetics, 3 mg/kg, iv dose, DAR 8 conjugates

B. ADC clearance as a function of PEG size

C. Single dose mouse tolerability

D. Single dose mouse tolerability
Figure 5

**CD19+ RL xenograft model of DLBCL**

- 3 mg/kg, single i.p. dose, DAR 8 conjugates

<table>
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<th></th>
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<tr>
<td>CD19-10</td>
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<td>(no PEG)</td>
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<tr>
<td>CD19-7</td>
<td>2/5</td>
<td>(PEG4)</td>
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<tr>
<td>CD19-6</td>
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<td>(PEG6)</td>
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<tr>
<td>CD19-5</td>
<td>4/5</td>
<td>(PEG12)</td>
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**CD19+ Raji xenograft model of Burkitt's lymphoma, single dose i.p.**

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<tr>
<td>CD19-5</td>
<td>3 mg/kg (PEG12)</td>
</tr>
<tr>
<td>CD19-4</td>
<td>0.33 mg/kg (PEG12)</td>
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</tbody>
</table>

**CD19+ WSU-DLCL2 xenograft model of DLBCL, single dose i.p.**

<table>
<thead>
<tr>
<th></th>
<th>Dose (PEG)</th>
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<tbody>
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Molecular Cancer Therapeutics

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