Peptide-Cleavable Self-immolative Maytansinoid Antibody–Drug Conjugates Designed To Provide Improved Bystander Killing

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Supporting Information

ABSTRACT: A new type of antibody–drug conjugate (ADC) has been prepared that contains a sulfur-bearing maytansinoid attached to an antibody via a highly stable tripeptide linker. Once internalized by cells, proteases in catabolic vesicles cleave the peptide of the ADC’s linker causing self-immolation that releases a thiol-bearing metabolite, which is then S-methylated. Conjugates were prepared with peptide linkers containing only alanyl residues, which were all L isomers or had a single D residue in one of the three positions. A D-alanyl residue in the linker did not significantly impair a conjugate’s cytotoxicity or bystander killing unless it was directly attached to the immolative moiety. Increasing the number of methylene units in the maytansinoid side chain of a conjugate did not typically affect an ADC’s cytotoxicity to targeted cells but did increase bystander killing activity. ADCs with the highest in vitro bystander killing were then evaluated in vivo in mice, where they displayed improved efficacy compared to previously described types of maytansinoid conjugates.

KEYWORDS: Antibody–drug conjugates, maytansinoid, peptide

Antibody–drug conjugates (ADCs) are composed of an antibody attached to a cytotoxic payload via a linker.1,2 Most ADCs bind to target antigens expressed on the surface of cancer cells, then the conjugate internalizes into catabolic vesicles where the antibody, and possibly the linker, are degraded to release one or more cytotoxic metabolites. In some cases, these released metabolite(s) are membrane permeable, allowing them to diffuse into and kill neighboring cells in a tumor, an effect known as bystander killing.3 ADCs have been investigated for more than two decades; however, ado-trastuzumab emtansine (Kadcyla), for the treatment of metastatic breast cancer, is currently the only conjugate approved for a solid tumor indication.4 It is a maytansinoid bearing ADC that has a noncleavable linker and releases a charged metabolite that does not induce bystander killing.1 The high stability of noncleavably linked ADCs is expected to maximize delivery of cytotoxic molecules to tumors while reducing payload release in healthy tissues.2 However, the linker may not be ideal as many other conjugates using the same linker and payload as ado-trastuzumab emtansine were not successful in the clinic.3 Also, based on mouse xenograft studies, cleavably linked conjugates that induce bystander killing are typically more efficacious than noncleavably linked ADCs.6,7

We have previously developed two types of maytansinoid ADCs that induce bystander killing, each have strengths but also potential for improvement. In the first type, the payload is linked to a lysine residue of an antibody via a disulfide bond, ADCs (1a–1d) are shown as examples, Figure 1A.8 After internalization by cells, the antibody of the ADC is catabolized to give 2 and most is reduced to give the thiol-containing maytansinoid (3a), some of which can then be S-methylated to 3b. Only 3a and 3b are hydrophobic, allowing them to diffuse into and kill bystander cells. Most of 3a that reaches the liver in vivo is S-methylated to 3b, which is efficiently oxidized to less potent compounds, potentially reducing systemic toxicity.1 We have previously shown that disulfide-linked ADCs typically show superior efficacy in mouse tumor xenograft models than conjugates bearing the same noncleavable linker used in ado-trastuzumab emtansine.5 The second type of maytansinoid conjugate that induces bystander killing has a peptide-anilino linkage, such as 4a–4c, Figure 1B.10 These ADCs are catabolized by cells to give the potent metabolite 5, which can induce bystander killing. However, linker half-lives of both disulfide-linked and anilino conjugates were only ~5.5 days,10 which compares poorly to the stability of noncleavably linked conjugates (9.3 day).11

ADCs can incorporate peptide linkers without anilino moieties; however, in order to maximize metabolite hydrophobicity, the conjugate should release noncharged compounds. Although their work was not on ADCs, Kingsbury and coauthors reported that a peptide could be enzymatically cleaved followed by immolation of the resulting hydrophilic amine to give a hydrophobic thiol-bearing compound.12 Based on these findings, the goal of this work was to design highly

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stable protease cleavable maytansinoid conjugates that release hydrophobic metabolites, capable of bystander killing, and determine if they are superior to disulfide-linked and anilino ADCs.

The humanized mAbs anti-huEGFR (targeting human epidermal growth factor 1),13 anti-huFRα (targeting human folate receptor-alpha),12 anti-huCanAg (targeting human CanAg antigen),15 and anti-huMet (targeting human cMet antigen)16 were used in these studies. New immolative ADCs 6a−6d (Ab = anti-huEGFR), 7c, 7e (Ab = anti-huFRα), 8a, 8c (Ab = anti-huCanAg), and 9c (Ab = anti-huMet) with tripeptide linkers were prepared, Figure 1C. After internalization into targeted Ag+ cells, the new ADCs were designed to be cleaved in lysosomes up to the immolative nitrogen atom, followed by release of the corresponding thiol-containing metabolite (11a or 11b), which in turn could potentially be S-methylated giving maytansinoids (12a or 12b). In order to simplify the interpretation of results, alanine was the only amino acid residue used in the linkers. Peptide linkers can often be stabilized by replacing an L-amino acid with its D-isomer.10,17 Thus, the effect of replacing one of the linker L-alanyl residues with its D isomer was investigated. Linker amino acids are listed in the direction from the antibody toward the maytansinoid, for example, (L, L, D) indicates that a D-alanyl residue is directly attached to the immolative nitrogen.

Increasing a molecule’s hydrophobicity typically improves its diffusion into cells.18 Therefore, we wished to determine if changing the number of hydrophobic methylene units in the maytansinoid side chain, n, would affect ADC bystander killing.

Immolative ADC payloads were prepared as depicted in Scheme 1. Maytansinol (13) was reacted with the carbonic acid group of DM followed by oxidation of the sulfur atoms of 12a−12c.

![Figure 1. (A) Structures of ADCs bearing disulfide linkers and the metabolites that are formed from their proteolysis in catabolic vesicles followed by disulfide cleavage and S-methylation. DM indicates the depicted maytansinoid moiety, derivatizable on the N-methyl alanylamino. (B) Structures of ADCs with peptide-anilino linkers and the metabolite that is formed from proteolysis. (C) Structures of the new ADCs bearing peptide-immolative linkers and the metabolites that are expected to be formed in catabolic vesicles followed by S-methylation. Bracketed letters (L or D) indicate alanyl stereochemistry in the direction from antibody toward the maytansinoid. (D) Structures formed if the sulfur atoms of 12a−12c are oxidized.](image)

### Scheme 1. Synthesis of New Immolative Maytansinoid ADCs

**Reagents and conditions:**
- (a) Zn(OTf)2, DIPEA
- (b) Pb(OAc)4, AcOH
- (c) HS-(CH2)n-COOH, TFA
- (d) EDC, 14
- (e) morpholine, 22c, 23.
anhydride of N-methyl alanine in the presence of zinc triflate and N,N-dissopropylethylamine (DPEA) to give 14.22 Fmoc-protected alanyl peptides (15a–15d) were prepared by solid phase synthesis,22 then oxidatively decarboxylated with lead tetracetate and acetic acid in dimethylformamide (DMF) to give 16a–16d respectively.23 Compounds 16a–16e were reacted with the appropriate thiol-bearing carboxylic acid in the presence of ∼20% trifluoroacetic acid (TFA) in dichloromethane to give 17a–17e. Compound 14 was coupled to 17a−17e using 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) to give 18a−18e, which were then deprotected with morpholine giving 19a−19e. The heterobifunctional linker (20) was coupled to 19a−19e to give 21a−21e, and disulfide reduction with 1,4-dithiothreitol (DTT) gave payloads (22a−22e). Immolative ADCs were prepared from one of these payloads and a mAb, as depicted for representative conjugations with 22c. Scheme 1. The heterobifunctional linker 23 was coupled to the thiol of 22c in a solution of buffer–organic solvent. The reaction solution, without purification, was reacted with a mAb, and the mixture was purified by size exclusion chromatography (SEC). Disulfide-linked and peptide anilino ADCs as well as their metabolites were prepared as previously described.10,22

The compounds that were expected to be formed when cells metabolize immolative ADCs were prepared as depicted in Figure 2. Compound 14 was reacted with 6-mercaptohexanoic acid to give 11a, which was then reacted with iodomethane to give 12a. Compound 14 was also coupled with 20 to give 2. The disulfide of 24 was then reduced with DTT to give 11b, which was reacted with iodomethane to give 12b. The S-methylated metabolites could potentially be oxidized on their sulfur atoms in liver. Representative compounds 12a′ and 12a″ were prepared by oxidizing 12a with tert-butyl hydroperoxide in the presence of vanadium acetylacetonate.

To determine if linker stereochemistry affected cytotoxicity, the anti-huEGFR immolative ADCs (6a−6d) were incubated with Ag+ CA922 cells, Figure 3A. Cytotoxicities for all ADCs were determined as previously described.23 Conjugate 6d (I1, I1, d1) was the least potent, while the other conjugates exhibited similar high cytotoxicities. This indicated that ADC cytotoxicity was impaired when a d-alanine was directly attached to the immolative nitrogen, which will be designated as a d-linked conjugate. Conjugate 1a also had similar potency as 6a−6c against CA922 cells. In each case, the addition of a 1000-fold molar excess of the unconjugated anti-huEGFR antibody fully inhibited ADC potency, demonstrating that cytotoxicity was antigen-dependent.

Next, the effect of methylene side chain length (n) on ADC cytotoxicity to cells that homogeneously express target antigen was investigated. The cytotoxicities of 7c and 7e, having n values of 3 and 5, respectively, were most often similar to each other, and to 1b (Supplementary Table S1). ADCs 6a−6d were tested for their ability to induce bystander killing in vitro, Figure 3B. A fixed number of Ag− cells were incubated with ADC and a variable number of Ag+ cells.3 The Ag+ cells take up and degrade the ADC to release metabolite(s) that can kill these cells. Membrane permeable metabolite(s) may also diffuse into and kill a portion of the proximal Ag− cells (bystander cells). ADCs that induce the highest level of bystander killing require addition of the lowest number of added Ag+ cells to kill the Ag− cells in the mixed cell population. The relative number of HCC827 Ag+ cells needed to kill 50% of the MCF-7 Ag− cells were compared. Conjugates 6d and 1a required addition of ∼1250 Ag+ cells, while the other ADCs required only ∼400 Ag+ cells. Thus, 6a, 6b, and 6c induced ∼3-fold greater in vitro bystander killing than 6d and 1a.

As shown in Figure 3C, increasing the number of methylene units in the maytansinoid side chain increased the in vitro bystander killing of immolative ADCs. Conjugates 7e and 7c, having 3 and 5 methylene groups in the maytansinoid side chain, required approximately 1800 and 700 Jeg-3 Ag+ cells,

Figure 2. Preparation of possible immolative ADC metabolites 12a, 12b, 12c, 12a′, and 12a″.
respectively, to induce 50% Nam/Luc Ag− cell killing. Thus, 7c induced ∼2.5-fold more bystander killing than 7e. Conjugate 7c also induced more bystander killing than 4b and 1b, which required approximately 950 and 2000 Ag+ cells, respectively, to induce 50% cell killing. The similar cytotoxicities of 7c and 7e could be explained if their metabolites are produced with similar efficiencies and have similar potencies once inside cells. However, if the metabolites of 7c are more hydrophobic, as expected, then they could more easily diffuse into other cells to induce more bystander killing. Additional studies will be needed to determine if this is the case; however, because of their lower bystander killing, conjugates with n = 3 were not evaluated further.

The metabolites of 8c were identified by incubating the conjugate with the high processing cell line COLO-205 for 24 h followed by cell lysis with organic solvent, using a previously described method. As a control, COLO-205 cells were treated the same way but without prior exposure to 8c. Both samples were analyzed by UPLC/MS (Supplementary Figure S1). The 252 nm UPLC trace of cells treated with 8c showed two peaks that were not in the control, a major peak at 12.73 min and a minor peak at 7.72 min having about a fourth of the area of the major peak. The synthesized S-methylated standards 12a and its sulfoxide 12a′, shown in Figure 1D, were found to have the same retention times and mass spectra as the 12.73 and 7.72 min peaks, respectively. Sulfoxides are prone to air oxidation, and trace metals can act as catalysts. Indeed aqueous solutions of standard 12a were oxidized to 12a′ over time (data not shown). Indicating that 12a′ in cell lysates was likely an artifact due to oxidation of 12a by air.

The in vitro cytotoxicities of the synthesized S-methylated metabolites 12a−12b were evaluated on the H1703, H1975, and COLO-704 cell lines, Table 1. The potency of 12a (n = 5) was consistently ∼2.5 times that of 12b (n = 3), indicating that the cytotoxicity of free metabolites increased as hydrophobic methylene units were added. Also, 12a had a similar potency to the isomeric compound 3b. The synthesized sulfone 12a″ and the racemc sulfoxide 12a′ were at least 80-fold less cytotoxic than the precursor 12a. Indicating that if 12a is efficiently oxidized in liver it should be substantially inactivated.

Earlier studies with maytansinoid ADCs indicated mouse tolerabilities between 1200−1500 μg/kg based on the mass of delivered payload. An initial study was conducted using anti-huEGFR ADCs (2 mice/group) at a dose of 1200 μg/kg based on the mass of delivered payload (47 mg/kg based on the mass of antibody) to determine if the tolerability of immolative conjugates was affected by having a single D-alanyl in the tripeptide linker and how these tolerabilities compared to a disulfide-linked ADC, Figure 4. The groups receiving vehicle, 1a, and 6c gained weight, although some skin reddening was noted in mice receiving 6c. In contrast, animals that had been treated with 6a and 6b lost weight and displayed pronounced skin reddening. The mice in the 6d group, d-linked conjugate, also displayed some skin toxicity, but weight loss was not as pronounced as the 6a and 6b groups. This study revealed that immolative ADCs bearing the L-Ala-D-Ala-L-Ala linker were better tolerated in mice than those bearing the other tripeptide linkers.

A second MTD study was undertaken, using ADCs bearing an anti-huFRα antibody, Figure 5A. The groups (8 mice/group) consisted of vehicle, 4b (1250 μg/kg), and the immolative ADC 7c (1d, 1l) at 1000 and 1250 μg/kg. Mice in all groups gained weight, but the 7c (1250 μg/kg) group had some skin reddening. Because of their favorable tolerability, immolative ADCs bearing the L-Ala-D-Ala-L-Ala linker were evaluated in further in vivo studies.

The clearance of 7c at a dose of 10 mg/kg from circulation in nontumor bearing mice was determined by ELISA as previously described. The half-lives of the ADC’s antibody and drug components were ∼15.2 and ∼10.9 days, respectively, Figure 5B.

The in vivo efficacy of maytansinoid ADCs 9c and 1d was compared in mice (8/group) bearing Hs 746T tumor xenografts, where the target c-Met antigen is expressed homogeneously, Figure 6A. The H-Score for these cells, a measure of antigen expression, is 160. At the 2.5 mg/kg dose, complete regressions (CRs) were achieved for all mice in the 9c group, but only two CRs were seen in the 1d group, which latter regrew. Next anti-huCanAg ADCs (5 mg/kg) were compared in mice (6/group) bearing HT-29 xenografts, where CanAg is only expressed on 20–30% of cancer cells in the tumor, Figure 6B. The greatest antitumor activity was observed with the immolative ADCs 8a (1d, 1l) and 8c (1l, 1d), respectively.
L), resulting in CRs in 6/6 mice, although tumors began to regrow at day 30. The anilino ADC 4c (2/6 CRs) and the disulfide-linked ADC 1c (0/6 CRs) were less efficacious. HT-29 xenograft bearing mice (6/group) were also treated at 2.5 mg/kg; group 8c (2 CRs) had a larger reduction in median tumor size than group 8a (1 CR), Figure 6C. The in vivo efficacy of anti-huFRα ADC was compared in mice bearing OV-90 xenografts, which express folate receptor heterogeneously (H-Score = 48), Figure 6D. The immolative ADC 7c was approximately twice as efficacious as the anilino ADC 4b, based on the similarity in the tumor volume plots for group 7c (1.25 mg/kg) and group 4b (2.5 mg/kg), and the similar or better response of group 7c (2.5 mg/kg) compared to group 4b (5 mg/kg).

In conclusion, the in vitro bystander killing of immolative ADCs positively correlated with metabolite hydrophobicity. Conjugates with adjacent linker l-alanyl residues were not as well tolerated and were less efficacious than the (l-Ala-l-Ala-l-Ala) peptide-linked ADC. We postulate that ADCs with adjacent l-alanyl residues may be less stable in vivo, which would increase metabolite release in healthy tissues, and lower payload delivery to tumors. Finally, increasing both in vitro bystander killing, and linker stability resulted in ADCs having a better therapeutic window in mice than previously described maytansinoid conjugates.

ASSOCIATED CONTENT

* Supporting Information
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Detailed experimental procedures(PDF)
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Notes

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Abbreviations

ADC, antibody-drug conjugate; CR, complete regression (tumor xenograft no longer detected); DIPEA, N,N-diisopropylethylamine; DMF, dimethylformamide; DTT, 1,4-dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; PR, partial regression (50% reduction of tumor xenograft size); Et3N, triethylamine; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid

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