Preclinical Characterization of Catabolic Pathways and Metabolism of ABBV-011, a Novel Calicheamicin-Based SEZ6 Targeting Antibody Drug Conjugate

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Catabolism and Metabolism of ABBV-011, a Calicheamicin ADC

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Abbreviations: ADC, Antibody-Drug Conjugate; ADME, Absorption, Disposition, Metabolism, and Excretion; DAR, Drug-Antibody Ratio; DRM, drug-related material; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FA, formic acid; GO, gemtuzumab ozogamicin; HER2, human epidermal growth factor receptor 2; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; IO, inotuzumab ozogamicin; LBA, ligand binding assay; LC+LD conjugated light chain with linker drug; LC-MS, liquid chromatography mass spectrometry; LD, linker drug; MS, mass spectrometry; MS², tandem mass spectrometry; MS³, second generation tandem mass spectrometry; PK, pharmacokinetic; S/N, signal-to-noise; SCLC, small cell lung cancer; SEZ6, Seizure Related 6 Homolog; TAb, total antibody; T-DM1, ado-trastuzumab emtansine; TmAb, total monoclonal antibody; UDPGA, uridine diphosphate glucuronic acid; UHR QTOF-MS, ultra-high resolution quadrupole time-of-flight mass spectrometer
Abstract

Antibody-drug conjugates (ADC) have gained momentum for treatment of cancers, with 14 ADCs currently approved for commercial use worldwide. (Fu, Li, Han, Shi, & Zhang, 2022) Calicheamicin is one of the payloads contributing to this trend, being utilized for both gemtuzumab ozogamicin (GO, trade name: Mylotarg) and inotuzumab ozogamicin (IO, trade name: Besponsa). Here, we discuss the catabolic pathway and metabolism of ABBV-011, a novel SEZ6-targeted, calicheamicin-based ADC being investigated for the treatment of small cell lung cancer (SCLC). Specifically, our investigation has found that disulfide bond cleavage in N-acetyl-γ-calicheamicin payload is a key liability that potentially impacts overall stability of the ADC. To our knowledge, there have been no reported observations of disulfide bond cleavage of calicheamicin ADCs. ABBV-011 utilizes a novel linker structure, leading to a distinct metabolic profile when compared to GO and IO. Despite this difference in linker structures, we propose that this liability may also be relevant for other calicheamicin ADCs. Multiple data sets supporting our investigation were acquired as part of the preclinical development of ABBV-011 and demonstrate the utility of in vitro experiments to characterize potential ADC candidates prior to clinical trials.
Significance Statement

Several *in vitro* and *in vivo* stability studies of ABBV-011, a calicheamicin-based ADC, identified circulating metabolites and catabolites and suggested that disulfide cleavage may be a key liability for the conjugated linker-payload. These observations may be relevant to other disulfide-linked ADCs, such as Mylotarg and Besponsa, both of which have reported similar half-lives that possibly indicate instability.
Visual Abstract

Introduction

ABBV-011 is a SEZ6-directed antibody-drug conjugate (ADC) consisting of SC17, a humanized cysteine engineered immunoglobulin 1 (IgG1) monoclonal antibody (mAb), that is conjugated in a site-specific manner to N-acetyl-γ-calicheamicin through a noncleavable linker (LD19.10) (drug-antibody ratio = 2). This novel linker is structurally unique, relying on disulfide cleavage for payload release, cysteine maleimide conjugation for attachment, and a PEGylated spacer between the attachment and release points. As a result, ABBV-011 releases a unique profile of calicheamicin-based catabolites, with the most abundant being cyclized cysteine-LD19.10 (hereafter referred to as LD19.10-M8b, or simply M8b). The structure is shown in Figure 1. (Wiedemeyer et al., 2022)

GO and IO both employ the same N-acetyl-γ-calicheamicin linker-drug for treatment of hematological cancers. Serious adverse events such as thrombocytopenia, neutropenia, and veno-occlusive disease have limited the utility of these agents. (Jen et al., 2018; Kantarjian et al., 2019; Kantarjian et al., 2016; Petersdorf et al., 2013) Three other calicheamicin ADCs, PF-06647263, (Garrido-Laguna et al., 2019) CMD-193, (Herbertson et al., 2009) and CMB-401, (Chan et al., 2003) all with the similar linker-drug as GO and IO, have been tested in clinical trials but were terminated for safety and/or efficacy reasons.

Calicheamicin contains a strained ring enediyne structure. It binds to the minor groove of DNA where the trisulfide is cleaved. The resulting thiol intramolecularly conjugates to the enediyne to relieve ring strain generating a reactive diradical; the Bergman cyclization. The interchelated diradical then abstracts hydrogen atoms from DNA, leading to cleavage of the DNA chain. (Walker, Landovitz, Ding, Ellestad, & Kahne, 1992)
GO has a total monoclonal antibody (TmAb) half-life of 72 hours (58% CV) and a total calicheamicin half-life of 45 hours (56% CV) in human for the first dose, (Dowell, Korth-Bradley, Liu, King, & Berger, 2001) and a TmAb half-life of 109 hours and a total calicheamicin half-life of 47 hours in mouse. This is consistent with IO which has a TmAb half-life of 67 hours (52% CV) and a total calicheamicin half-life of 17 hours (29% CV) in human (Advani et al., 2010) with a TmAb half-life of 55 hours and a total calicheamicin half-life of 29 hours in mouse. (Boghaert et al., 2008) All of the calicheamicin ADCs where pharmacokinetic (PK) data are available point to a separation of the TmAb and ADC PK profiles suggesting instability of the calicheamicin ADC in circulation.

GO and IO both appear to be relatively stable (<10% cleavage) in rat, monkey, or human plasma or in the presence of serum levels of GSH up to 96 hours. (European Medicines Agency, 2018) Yet under conditions associated with intracellular compartments such as low pH (4.5) or intracellular GSH levels, linker cleavage is more facile. (European Medicines Agency, 2018) This may imply that the in vivo drug-antibody ratio (DAR) changes observed with GO and IO may partially result from linker cleavage during cellular recycling of the biologic.

The only measured metabolite from either IO or GO was the N-acetyl-γ-calicheamicin DMH fragment, of which metabolism has been reported to be primarily disulfide reduction. (European Medicines Agency, 2017, 2018) Liver S9 metabolic studies suggest that a majority of disulfide reduction occurs non-enzymatically. (European Medicines Agency, 2018) No information is available on whether any steps were taken to preserve stability of the linker despite including a disulfide-bond. It has been suggested that the AcButDMH linker can be found to hydrolyze slowly in the systemic circulation, resulting in release of payload and off-target toxicity. (Fu, Li, Han, Shi, & Zhang, 2022) Another possibility is cleavage of the disulfide bonds to release the payload. ADC linker disulfide bond reduction has been shown to be catalyzed by thioredoxin.
and glutaredoxin, two common oxidoreductase enzymes present in the blood. (Zhang et al., 2019)

It has been suggested that the high \textit{in vivo} aggregation of GO may be due to AcButDMH linker hydrophobicity. Consistent with this hypothesis, Genentech has reported ADCs with “linkerless” payload attachment lower the aggregate level which correlates to an observed total antibody half-life of 24 days and an observed conjugated ADC half-life of 21 days in mouse. (Vollmar et al., 2021)

During the development of ABBV-011, the ligand binding assay (LBA) analysis of \textit{in vivo} cyno PK samples observed a disconnect between total antibody (TAb) and conjugated ADC concentrations (Figure 2, Supplemental Table 1) consistent with previous observations with GO and IO. This triggered a series of \textit{in vitro} and \textit{in vivo} experiments to understand the mechanism of this instability. Here, we discuss the results of these experiments, including characterization of \textit{in vitro} plasma stability by high-resolution mass spectrometry (HRMS) of the intact ADC, as well as analysis of payload metabolites released in plasma, lysosome, and liver S9 matrices. These \textit{in vitro} observations correlated well with \textit{in vivo} ABBV-011 cyno toxicokinetic studies and likely are involved with the differential exposures of TAb and ADC concentrations for GO and IO.
Methods

In Vitro Liver S9 Metabolism

M8b and ABBV-011 liver S9 incubations were conducted with male Sprague-Dawley rat (Celsis In Vitro Technologies), male cynomolgus monkey (Celsis In Vitro Technologies), or mixed-gender pooled human (BD Gentest) S9 fractions. M8b was provided by Abbvie Medicinal Chemistry with a purity of >90%, as determined by liquid chromatography mass spectrometry (LC-MS) and UV detection.

Incubations were conducted for 0 and 1 hour, with cofactors. A 48 hour incubation was also conducted for ABBV-011. A standard 200 µL incubation mixture was prepared by initial incubation of alamethicin (final concentration 10 µg/mL) and individual liver S9 fractions (final concentration 2 mg/mL) on ice for 15 minutes. A mixture of 100 mM potassium phosphate buffer at pH 7.4, MgCl₂ (final concentration 3.3 mM) and water were then added, followed by addition of the substrate (M8b: final concentration 5 µM M8b, ABBV-011: final concentration 2 mg/mL). The reaction was initiated by adding a cofactor mixture of NADPH (final concentration of 1 mM), uridine diphosphate glucuronic acid (UDPGA) (final concentration of 5 mM), and GSH (final concentration of 5 mM). Incubations were conducted at 37°C, after which organic solvent (M8b: 400 µL acetonitrile/methanol mixture (1/1, v/v); ABBV-011: 200 µL of cold methanol was added, followed by addition of 400 µL of cold acetonitrile) was added to stop the reaction. The mixtures were vortexed and then centrifuged for 15 minutes at 4000 rpm and 4°C. The ABBV-011 supernatant was removed, dried under nitrogen, and resuspended in 100 µL of water prior to injection on LC-MS. The M8b supernatant injected directly on LC-MS.

Liver S9 extracted samples were loaded on a Phenomenex Kinetex C18 2.6 µm, 100 x 4.6 mm i.d. high performance liquid chromatography (HPLC) column, and metabolites were eluted at 40°C using an HPLC system consisting of an UltiMate 3000 RS Pump and UltiMate 3000 RS
Autosampler. Samples were analyzed using a Orbitrap Q Exactive Plus Mass Spectrometer (ThermoFisher Scientific) equipped with an electrospray ionization (ESI) source operated in the positive ion mode. The Orbitrap Q Exactive was operated with mass resolution at 70000 for full scan and 17500 for tandem mass spectrometry (MS²) scan. Data was manually analyzed using Thermo Xcalibur software v 4.0.

**In Vitro Lysosomal Catabolism**

Human liver lysosomes (Xenotech LLC, H0610.L) and rat liver tritosomes (Xenotech LLC, R0610.LT) were resuspended in 50 mM ammonium acetate buffer, pH 5, with a final protein concentration of 0.25 mg/mL. ABBV-011 was spiked into lysosomal mixtures at a final concentration of 50 µg/mL and incubated at 37°C for 0, 2, 4, 24 and 48 hours, respectively. At each time point, 100 µl of cold methanol was added into 100 µl of sample, followed by 200 µl of acetonitrile, to stop the reaction. The solutions were centrifuged for 15 minutes at 3300 rpm (1820 x g) and the supernatant was removed and stored at -20°C until the samples were analyzed.

The components in the *in vitro* samples were profiled following separation by HPLC coupled to a HRMS. The HPLC system consisted of a Dionex HPG-3200RS Pump and a Dionex WPS-3000 Autosampler. The elution of catabolites was achieved at 50°C on a Phenomenex Luna Omega 1.6 µm Polar C18, 100 Å, 150 x 2.1 mm i.d. HPLC column. The mobile phases were A: 0.1% formic acid in water, and B: 0.1% formic acid in acetonitrile; the flow rate was maintained at 0.4 mL/min. A gradient of 5-95% mobile phase B was applied over 45 minutes.

Characterization of ABBV-011 catabolites was performed by mass spectrometry using an Orbitrap Elite Mass Spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a heated electrospray ionization (HESI) source operated in the positive ion mode. The instrument was operated with mass resolution at 120,000 for full scan and 15000 for MS² and 2nd
generation product ion spectra (MS^3) scan. Typical mass errors of analytes relative to theoretical masses are less than ± 10 parts per million in daily operations. Mass spectrometry (MS) data were processed using Thermo Xcalibur 4.0.

**ABBV-011 Small Molecule In Vitro Plasma Metabolism**

ABBV-011 was spiked into human (Bioreclamation, HMPLEDTA2), cynomolgus monkey (Bioreclamation, CYNPLEDTA2) and rat plasma (Bioreclamation, RATPLEDTA2) at a final concentration of 300 µg/mL and incubated at 37°C for 0, 3, 24, 48 and 72 hours. At each time point, 100 µl of cold methanol was added into 100 µl of sample, followed by 200 µl of acetonitrile, to stop the reaction. The solutions were centrifuged for 15 minutes at 3300 rpm (1820 x g) and the supernatant was removed and stored at -20°C until the samples were analyzed.

LC-MS analysis of plasma samples followed the same workflow as analysis of lysosomal samples, as described above.

**ABBV-011: In Vitro and In Vivo Cyno Plasma Stability**

ABBV-011 was spiked in cyno plasma at 50 µg/mL concentrations and incubated at 37°C for 7 days in order to assess *in vitro* plasma stability. Various time points were collected from 0 h, 24 h, 72 h, and 168 h, respectively. Post incubations, all the time points were subjected to two-step immuno-affinity enrichment on an Assaymap Bravo robotic platform. For assessing the *in vivo* stability of ABBV-011, 50 µL of *in vivo* cyno study plasma samples were subjected to same sample prep as the *in vitro* incubated samples. The first step involved immobilization of the biotinylated anti-human IgG capture reagent on to the streptavidin cartridges, followed by the second step of affinity purification of the samples. The eluted samples were injected (without further processing or after reduction of disulfides) on a Thermo U3000 LC system connected to a Bruker maXis II ultra-high resolution quadrupole time-of-flight mass spectrometer (UHR
QTOF-MS) system. The analysis was conducted using a porous R2 column (10 µm, 2.1 x 30 mm) with 0.1% (v/v) formic acid (FA) in water as mobile phase A, and 0.1% (v/v) FA in acetonitrile as mobile phase B. A gradient of 10-40% mobile phase B was applied over 10 minutes at 300 µL/min. The MS was operated under denaturing conditions using a m/z range of 300-3500 and a capillary voltage of 4.5 kV. The MS data was analysed within the Bruker BioPharma Compass software using the maximum entropy deconvolution algorithm. Peak intensities of deconvoluted light chain and heavy chain (or dimers for non-reduced samples) with or without the linker drug were recorded for all time points. In addition, masses corresponding to the unconjugated species as well as linker-drug clippings/degradation were noted and monitored. The percent conjugate remaining at each time point was calculated in reference to the 0 h time point, with an assumption of time zero values being 100%. The assay imprecision was +/- 25%.

Sprague-Dawley Rat and Cynomolgus Monkey In Vivo Metabolism

Equal volumes of rat plasma samples (250 µl) were pooled from animals (n = 3) per time point: T1 = 5 min; T2 = 24 h; T3 = 336 h. For each pooled sample, 150 µl of cold methanol was added to 150 µl of pooled plasma, followed by adding 300 µl of acetonitrile to the mixture. The solutions were centrifuged for 15 minutes at 3300 rpm (1820 x g) and the supernatant was collected and stored at -20°C until the sample was analyzed by LC-MS.

Cynomolgus monkeys were given two intravenous doses (separated by 21 days) of 15 mg/kg ABBV-011. For cycle 1, plasma samples were collected from individual animals (6001 and 6002) at the following time points: 5 min, 6 h, 24 h, 48 h, 72 h, 96 h, and 168 h. The equal volume of plasma samples (1.5 mL) were pooled at three different time points: T1 (5 min - 24 h); T2 (48-72 h); T3 (96-168 h). For cycle 2 (week 3), plasma samples were pooled from both animals (n = 2) per time point: T1 = 5 min; T2 = 6 hr; T3 = 24 hr; T4 = 72 hr; T5 = 168 hr. For
each pooled sample, 150 μl of cold methanol was added to 150 μl of pooled plasma, followed by adding 300 μl of acetonitrile to the mixture. The solutions were centrifuged for 15 minutes at 3300 rpm (1820 x g) and the supernatant was collected and stored at -20°C until the sample was analyzed.

LC-MS analysis of plasma samples followed the same workflow as analysis of lysosomal samples, as described above.

Radiolabeled Rat In Vivo Metabolism

N-acetyl-γ-calicheamicin was tritium radiolabeled and conjugated to the SC17 mAb. The structure of ABBV-011 tritium-labeled linker-payload is displayed in Supplemental Figure 1.

Intact rats received a 23.75 mg/kg intravenous dose comprising [3H] ABBV-011 and nonradiolabeled ABBV-011, with a target radioactivity of 100 μCi/rat for IV dosing. Plasma samples were collected at 6, 24, 48, and 72 hours post dose.

A global time-point weighted AUC pooling method was used to pool rat plasma samples from 0-72 h. Following pooling, pooled plasma samples were mixed 1:1 with methanol and vortexed, after which they were mixed 1:1 with acetonitrile, vortexed, and centrifuged at 3220 x g at 4°C for 30 minutes. The supernatants were partially dried under nitrogen and then loaded on a Kinetex C18, 2.6 μm, 100 Å, 100 x 4.6 mm i.d. HPLC column. Following separation by HPLC, the eluant was split between a Q Exactive Plus Mass Spectrometer for structural identification and an Agilent 1200 series fraction collector equipped with Deepwell Luma Plate™-96. Radioactivity was counted using a Perkin Elmer Microplate Scintillation Luminescence Counter Topcount NXT.

Characterization of ABBV-011 and circulating metabolites was performed by mass spectrometry using Orbitrap Q Exactive Plus Mass Spectrometer (ThermoFisher Scientific, San Jose, CA).
equipped with an ESI source operated in the positive ion mode. The instrument was operated with mass resolution at 70000 for full scan and 17500 for MS² scan. Typical mass errors of analytes relative to theoretical masses are less than ± 5 parts per million in daily operations. MS data were processed using Thermo Xcalibur 4.0.

Additional information on all *in vivo* animal studies discussed in this publication is presented in Supplemental Table 2.
Results

Several of the same metabolites, albeit in different ratios, were observed across various *in vitro* and *in vivo* assays conducted to understand the metabolism of ABBV-011. Table 1 provides a summary of these observed metabolites. Major metabolites are presented in Figure 3, while minor metabolite structures are shown in Supplemental Figure 2.

The major *in vivo* circulating metabolites were cyclized cysteine-LD19.10 (M8b) and its linear isomer (M8a), and the N-acetyl-ε-calicheamicin product of the Bergman cyclization (M1) and its O-linked deglycosylated tetra-saccharide metabolite (M3). M1 and M3 were confirmed to be inactive metabolites.

*In Vitro* Liver S9 Incubation

Investigation of the *in vitro* metabolism of M8b in monkey and human liver S9 systems and ABBV-011 in rat and human liver S9 systems identified several metabolic pathways. The metabolic pathways identified for M8b involved reduction of the disulfide (M1), enzymatic O-deglycosylation of the tetra-saccharide followed by subsequent dehydrogenation (M4), and oxidation of the dehydrogenated tetra-saccharide (M9). Rearrangement of cyclic M8b to linear M8a was also observed. All metabolites observed after incubation with human liver S9 were also observed after monkey liver S9 incubation.

Metabolic pathways identified for ABBV-011 involved reduction of the disulfide (M1), enzymatic O-deglycosylation of the tetra-saccharide (M3 and M5), and enzymatic O-deglycosylation of the tetra-saccharide followed by subsequent dehydrogenation (M4). M5 was only observed after incubation with human liver S9, while all other metabolites were observed after both human and monkey liver S9 incubation.
ABBV-011: *In Vitro* Lysosome and Plasma Small Molecule Metabolism

The *in vitro* metabolism of ABBV-011 was investigated using lysosome (human and rat) and plasma (rat, cynomolgus monkey, and human). Following 48 hour incubation of 50 μg/mL of ABBV-011 in both human liver lysosomes and rat liver tritosomes at 37°C, two catabolites were observed, including M8a and NRGEC-capped LD19.10 (M6). Following 72 hour plasma incubation of 300 μg/mL of ABBV-011 at 37°C, five metabolites were observed in human plasma, while rat and monkey plasma exhibited six metabolites each. The metabolic pathways in cross species plasma incubation primarily involve retro-Michael addition (M8b), disulfide reduction (M1), hydrolysis of cysteine-LD19.10 (M2), O-linked deglycosylation (M3), subsequent dehydrogenation (M4), and reduction (M7). Qualitatively similar profiles were observed for ABBV-011 in cross species plasma incubations. There was no human-specific metabolite identified in lysosome and plasma incubation.

**ABBV-011: *In Vitro* and *In Vivo* Cyno Plasma Stability**

Stability of ABBV-011 was investigated *in vitro* by incubating the ADC in cyno plasma up to 7 days and *in vivo* using the cyno study samples, thereby immuno-purifying the ADC from matrix and analyzing in high-resolution-TOF MS system (as indicated in the method section). Intact mass analysis of both the datasets observed a major conjugated metabolite (CM1) as a result of hindered disulfide reduction on the light chain conjugated linker drug (LD) thereby releasing the calicheamicin-SH payload as the free thiol (Figure 4). Over time CM1 converted to CM2 as a result of cysteinylation of the free cysteine after the disulfide bridge breakdown. An increase in CM2 was observed over time both *in vitro* and *in vivo*. Overall decrease in the conjugated light chain with linker drug (LC+LD) as well as unconjugated (free) light chain signal was observed along with an increase in cysteinylated light chain over time. Other minor (trace level)
conjugated LD clippings were observed as a result of partial/full loss of sugars (CM3, CM4, and CM5) as shown in Figure 5.

These results indicate that the main reason for the instability of the N-acetyl-γ-calicheamicin linker drug is the breakdown of the hindered disulfide.

**Cynomolgus Monkey and Sprague-Dawley Rat In Vivo Metabolism**

The metabolite profiling of ABBV-011 was investigated in pooled plasma following intravenous administration of ABBV-011 in Sprague-Dawley rat (single dose at 80 mg/kg) and cynomolgus monkey (Q3 week x 2 doses at 15 mg/kg). Four metabolites (M1, M3, M4 and M8b) were observed in rat plasma, and two metabolites (M3 and M8b) were observed in cynomolgus monkey plasma. Similar metabolite profiles were observed in pooled monkey plasma after single dose and repeated dose. M8b was the only warhead-containing metabolite of ABBV-011 identified in monkey plasma.

**Radiolabeled Rat In Vivo Metabolism**

Two major circulating metabolites were identified as M8b and M1. Radio profiling demonstrated that M8b represented 63.2% drug-related material (DRM), while M1 represented 24.9% DRM in circulation. Due to the use of a split-flow for collection of fractions for radioanalysis, MS sensitivity was limited. Consequently, the ABBV-011 metabolites detected in non-radiolabeled monkey and rat samples were not detected by LC-MS analysis of the radiolabeled samples. To supplement radiochromatography data, synthetic M8b, M1, and diamine-calicheamicin standards were analyzed by LC-MS with identical chromatography parameters in order to confirm structures by matching retention times. The retention times of M8b and M1 matched the major peaks observed in the radiochromatogram, while diamine-calicheamicin eluted at an earlier retention time than any observed peaks.
In addition, synthetic M8b was spiked into plasma, processed by solvent extraction, and 7.6 pmol was loaded on column for LC-MS analysis, resulting in detection of M8b with 33 signal-to-noise (S/N). Radioactivity analysis of the pooled plasma sample suggests that 0.7 pmol M8b was loaded on column, more than an order of magnitude less than the synthetic M8b sample. Therefore, M8b and other related metabolites are estimated to be below LOD in the pooled plasma sample.

The retention times observed and calculated molecular ion masses, and mass error for the synthetic standards are included in Supplemental Table 3. The MS² spectra of M8b, M1, and M3 are available in Supplemental Figure 3. Some minor metabolites at low to trace levels are not included in this discussion and are unknown drug related material.
Discussion

These experiments revealed that disulfide bond cleavage is a significant factor accounting for loss of conjugated N-acetyl-γ-calicheamicin and a resulting reduction in DAR. This highlights the importance of carefully monitoring the stability of the disulfide bonds in other calicheamicin ADCs and preclinical ADCs. It may also suggest that linker designs that do not rely on disulfide bonds may be a more stable alternative for ADC design.

We found little information on the stability of the DMH-calicheamicin disulfide bond or the mechanism by which payload is released from GO or IO. The general assumption is that the acid-labile linker is hydrolyzed slowly over time at plasma pH. (Fu et al., 2022)(Wolf et al.)(Jabbour, Paul, & Kantarjian, 2021; Su et al., 2021) However, our data would suggest disulfide cleavage is the leading mechanism for release.

Conceptually, ABBV-011 internalizes and degrades in the lysosome to release calicheamicin-SH, M8a, or M8b. Calicheamicin-SH, M8a, or M8b would then enter the nucleus and bind to DNA. M8a or M8b would then be reduced to the common calicheamicin-SH intermediate, which would then undergo Bergman cyclization generating the diradical which would lead to cleavage of the DNA and cell-death. Detection of M1 does not necessarily verify the Bergman cyclization occurred interchelated to the DNA,(Myers, Cohen, & Kwon, 1994) but detection of M1 is required to verify successful cleavage of the DNA and efficacy of the ADC. The presence of M8a or M8b however, is indicative of a failure of some of the ADC to generate DNA scission.

Our results suggest that in vivo, a dominant pathway for ABBV-011 degradation in plasma involves reduction of the disulfide bond in the linker-drug, followed by cysteinylation of the linker (Figure 6). When ABBV-011 is incorporated into a tumor cell, it is transported to the lysosome, upon which the ADC is cleaved and calicheamicin material is released. Formation of M8a is favored over M8b in the lysosome due to the acidic conditions. M6 is also released in the
lysosome. *In vivo* efficacy demonstrates that the lysosome is also responsible for ultimate appearance of active forms of calicheamicin in the nucleus (disulfide clipped or forms released by disulfide bond reduction of M8b) and induces double-strand DNA scission and cell death.

Some ABBV-011 molecules are removed from circulation by the liver, upon which M1 and M3 are released, suggesting that ABBV-011 may be releasing active calicheamicin which may damage liver cells and cause toxicity. These observations are supported by *in vivo* cyno plasma metabolite analysis, which identified M8b and M3 in circulation.

Minimal toxicity was observed with dosing of the M8b metabolite to rats, suggesting that M8b must not be entering the nucleus, intercalating with DNA, and cleaving the DNA via Bergman cyclization diradical. One possible explanation is that M8b is poor at permeating the nuclear membrane. It’s also unlikely that M8b was generated extracellularly since it’s more likely the disulfide would clip in this environment, as demonstrated by our characterization of the intact ADC in plasma (Figure 4). Therefore, DNA cleavage is likely driven by calicheamicin-SH release via a mechanism independent of M8b formation.

We have also learned that N-acetyl-ε-calicheamicin (M1) is unstable and further metabolizes to release M3. N-acetyl-ε-calicheamicin has not been reported as a catabolite of GO or IO; although it has been stated that N-acetyl-ε-calicheamicin could not be separated from N-acetyl-γ-calicheamicin using a legacy enzyme-linked immunosorbent assay (ELISA) bioanalytical method. (Kirkovsky, 2016) The only published step taken to stabilize calicheamicin ADCs is optimization of the GO conjugation process to limit aggregation of the final product for clinical use. (Hamann et al., 2002) This resulted in a product with a range of DAR with an average of 2-3, with approximately 50% of the antibody unconjugated. (Bross et al., 2001) It is unclear if this observed propensity for aggregation is related to instability of the payload molecule.
We conclude that any measurements of an active calicheamicin species such as M1 should include measurements of the corresponding tetra-saccharide molecule such as M3, being mindful of its Absorption, Distribution, Metabolism, and Excretion (ADME) properties, for an accurate portrayal of how effectively the ADC is releasing calicheamicin and cleaving DNA. Our initial radiolabeled ABBV-011 rat experiments appeared to suggest that M8b is 2.5x more abundant than M1 in circulation. However, as M3 was not resolved in this LC-MS analysis, this ratio could not be accurately assessed. Future characterization of ABBV-011 should prioritize optimal chromatographic resolution of M8b, M1, and M3.

These results suggest that more work must be done to understand how calicheamicin is trafficked across the cell, from the lysosome, and into the nucleus. One example of this type of investigation were conducted on ado-trastuzumab emtansine (T-DM1, trade name: Kadcyla), an ADC indicated for treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer. (Verma et al., 2012) While Kadcyla is conjugated with DM1 rather than calicheamicin, it merits mention in this discussion as one of the key ADC studies that has looked at trafficking of the drug of an ADC. (Liang, Mei, Gao, Peng, & Zhan, 2021; Sang et al., 2021)

During the course of this work, it was discerned that N-acetyl-γ-calicheamicin is light sensitive. Protecting ABBV-011 and unconjugated linker-drug from light exposure were found to correlate with reducing aggregation, maintaining in vitro cell killing potency, and stabilizing the linker disulfide bond. Fluorescent light was found to have cause fewer liabilities than natural light, indicating that N-acetyl-γ-calicheamicin is most sensitive to light wavelengths of <400 nm. These precautions to protect ABBV-011 samples from light played an important role in the response to early observations of a disconnect between TAb and conjugated ADC PK in vivo. Light protective precautions were included for subsequent in vivo bioanalysis, to improve the overall stability of ABBV-011. This sequence of events suggests that other calicheamicin ADC
studies should consider similar precautions or take steps to rule out light sensitivity as a source of instability for their ADCs.
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Figure 6 and the visual abstract were created with BioRender.com.
Data Availability Statement

The authors declare that all the processed data supporting the findings of this study are available within the paper and its Supplemental Information. Raw data is available on request from the corresponding author.
Authorship Contributions

Participated in research design: Ladror, Gu, Tong, Schammel, Gavrilyuk, and Sarvaiya.

Conducted experiments: Ladror, Gu, and Sarvaiya.

Performed data analysis: Ladror, Gu, and Sarvaiya.

Provided study materials: Schammel and Gavrilyuk.

Wrote or contributed to the writing of the manuscript: Ladror, Gu, Haight, and Sarvaiya.
References


Footnotes

AbbVie sponsored and funded the study; contributed to the design; participated in collection, analysis, and interpretation of data; and in writing, reviewing, and approval of the final version.

All authors are employees, former employees or retirees of AbbVie and may own AbbVie stock. VT is currently an employee at Chinook Therapeutics. AS is currently an employee at Maze Therapeutics. JG is currently an employee at Deep Valley Labs. AH is currently an employee at Cannula Consulting. The external authors have no conflict of interest to report.

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Figure Legends

**Figure 1.** Structure of ABBV-011. ABBV-011 is designed to stoichiometrically contain 2 molecules of linker drug (LD19.10) per molecule of antibody (SC17). Only 1 linker-drug molecule is shown above to illustrate conjugation.

**Figure 2.** Total Ab and ADC profiles from *in vivo* cynomolgus monkey toxicokinetic study. Animals were dosed at 9 mg/kg.

**Figure 3.** ABBV-011 *in vitro* catabolic pathway

**Figure 4.** A) Intact mass analysis of ABBV-011 *in vivo* cynomolgus monkey plasma stability study samples. B) Structure of LD19.10 after disulfide reduction. C) An increase in S-S breakdown products *in vivo* was observed over time.

**Figure 5.** ABBV-011 conjugated metabolites identified in cynomolgus monkey plasma by intact mass analysis.

**Figure 6.** Transport of ABBV-011. A: ADC binds target. B: ADC internalizes and enters endosome and lysosome. C: M8b, calicheamicin derivative. D: Active enediyne – SH analog of active calicheamicin, generated via disulfide reduction or enzymatic reaction. E: Active enediyne enters nucleus and performs DNA scission.
Table 1. Combined metabolite table

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Name</th>
<th>ADC19.10 Lysosomal Incubation (Rat/Human)</th>
<th>ADC19.10 Plasma Incubation (Rat/Cyno/Human)</th>
<th>ADC19.10 S9 Incubation (Rat/Human)</th>
<th>M8b S9 Incubation (Rat/Human)</th>
<th>In-Vivo Cyno Tox</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-ε-calicheamicin (no sugar)</td>
<td>M1</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Hydrolyzed Cys-LD19.10</td>
<td>M2</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Tetra-saccharide</td>
<td>M3</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dehydrogenated Tetra-saccharide</td>
<td>M4</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>N-acetyl-ε-calicheamicin (no sugar)</td>
<td>M5</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>NRGEC-LD19.10</td>
<td>M6</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Reduced N-acetyl-ε-calicheamicin (O)</td>
<td>M7</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cys-LD19.10</td>
<td>M8a</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cyclic-Cys-LD19.10</td>
<td>M8b</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Oxidized Tetra-saccharide</td>
<td>M9</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Dehydrated N-acetyl-ε-calicheamicin (-H₂O)</td>
<td>M10</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Diamine-calicheamicin</td>
<td>-</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
Supplemental Data

Supplemental materials available in Supplemental Data pdf file.
Figure 1

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3

ABBV-011 LD19.10 (Structure shown)

Hydrolysis
Rat/Cyno/Hu IVPS

Hydrolyzed Cys-LD19.10

Rat Lysosome
Hu Lysosome

M8a
Linear Cys-LD19.10

M8b
Cyclic Cys-LD19.10

Disulfide reduction
Rat/Cyno/Hu IVPS

M3
Tetra-saccharide

M1
N-acetyl-α-calicheamicin

M1
N-acetyl-α-calicheamicin

Figure 3
Figure 4

(A) Intens x10^4

LC  LC+Cysteinilation
LC+GSH  '24461.0808

LC+LD-1329 (CM1) (S-S breakdown)

'23815.69 '23999.73 '23815.68 '23815.67

'24461.0808 '24462.0841 '24598.08 '24598.08

CM2  LC+LD-1329+ Cysteinilation+18

'23815.68 '23999.73 '23815.67 '23815.67

'24461.0808 '24462.0841 '24598.08 '24598.08

(B) LC+LD-1329 (CM1)

(C) In-vivo Cyno tox: LC+LD S-S break and Cysteinlation

5 min

'25790.37 '25591.26 '25808.37 '25808.39

LC+LD  LC+LD+18 Maleimide hydrolysis

CM4,CM5 '24598.08 '24598.08 '24598.08

CM3 '25609.28 '25609.26 '25609.27

24 hr

'25591.26 '25808.37 '25808.39

72 hr

'25808.39 '25808.39 '25808.37

168 hr

'25609.27 '25609.27 '25609.27
Figure 5

- **CM3**: LD19.10 (loss of sugars)
  - Trace

- **CM2**: LD19.10 (Disulfide Reduction, Cysteinylation)
  - Major

- **CM1**: LD19.10 (Disulfide Reduction)
  - Major

- **CM4**: LD19.10 (Partial Loss of Sugars)
  - Trace

- **CM5**: LD19.10 (Partial Loss of Sugars)
  - Trace

**ABBV-011**: LD19.10 (Structure shown)
Figure 6

- **Extracellular Space**

- **Active Enediyne**
  - **Cytoplasm**
    - **Nucleus**
      - **Calicheamicin derivative**
        - **pH~6 Endosome**
          - **pH~4 Lysosome**

The diagram illustrates the processes involving extracellular space, active enediyne, cytoplasm, nucleus, and the internal compartments of the cell with specific pH values.
Cycle 1 TK Parameters (Mean ± SD; n=3)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>AUC_{0-21d} [day·μg/ml]</th>
<th>Cmax [μg/ml]</th>
<th>t1/2 [day]</th>
<th>Clearance [mL/day/kg]</th>
<th>Vss [mL/kg]</th>
<th>TAb/ADC</th>
<th>Accumulation Ratio (AUC_{0-7d})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC</td>
<td>ADC</td>
<td>ADC</td>
<td>ADC</td>
<td>ADC</td>
<td>ADC</td>
<td>ADC</td>
<td>ADC/AUC_{0-21d} Cycle 2/Cycle 1</td>
</tr>
<tr>
<td>9</td>
<td>883</td>
<td>1604</td>
<td>311</td>
<td>280</td>
<td>4.5</td>
<td>13.8</td>
<td>9.9/3.7 1.8</td>
</tr>
</tbody>
</table>
**TC17-078, Cynomolgus Monkey**

<table>
<thead>
<tr>
<th>Group</th>
<th># of animals</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>ROA</th>
<th>Dose Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1M, 1F</td>
<td>ABBV-011</td>
<td>9</td>
<td>IV bolus</td>
<td>Q3wkx2</td>
</tr>
</tbody>
</table>

TK time points: Pre-dose, 5m, 6h, 24h, 72h, 168h, 336h, 504h post-dose #1; 5m, 6h, 24h, 72h, 168h post-dose #2
Samples analyzed by *In Vivo Cyno Plasma Stability Assay*

**TC17-215, Cynomolgus Monkey**

<table>
<thead>
<tr>
<th>Group</th>
<th># of animals</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>ROA</th>
<th>Dose Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2F</td>
<td>Vehicle</td>
<td>0</td>
<td>IV bolus</td>
<td>Q3wkx2</td>
</tr>
<tr>
<td>2</td>
<td>3F</td>
<td>ABBV-011</td>
<td>15</td>
<td>IV bolus</td>
<td>Q3wkx2</td>
</tr>
</tbody>
</table>

TK time points: Pre-dose, 5m, 6h, 24h, 72h, 168h, 336h, 504h post-dose #1; 5m, 6h, 24h, 72h, 168h post-dose #2
Samples analyzed by Cynomolgus Monkey *In Vivo Metabolism Assay*

**TOX133, Sprague-Dawley Rat**

<table>
<thead>
<tr>
<th>Group</th>
<th># of animals</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>ROA</th>
<th>Dose Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3M</td>
<td>Vehicle</td>
<td>0</td>
<td>IV bolus</td>
<td>once</td>
</tr>
<tr>
<td>2</td>
<td>3M</td>
<td>ABBV-011</td>
<td>10</td>
<td>IV bolus</td>
<td>once</td>
</tr>
<tr>
<td>3</td>
<td>3M</td>
<td>ABBV-011</td>
<td>30</td>
<td>IV bolus</td>
<td>once</td>
</tr>
<tr>
<td>4</td>
<td>3M</td>
<td>ABBV-011</td>
<td>50</td>
<td>IV bolus</td>
<td>once</td>
</tr>
<tr>
<td>5</td>
<td>3M</td>
<td>ABBV-011</td>
<td>80</td>
<td>IV bolus</td>
<td>once</td>
</tr>
</tbody>
</table>

Groups 1-4 TK time points: 5m, 24h, 72h, 120h, 168h, 336h post-dose
Group 5 had sparse TK sampling: 5m, 24h, 336h post-dose
Samples analyzed by Sprague-Dawley Rat *In Vivo Metabolism Assay*

**V18-023, Sprague-Dawley Rat**

<table>
<thead>
<tr>
<th>Group</th>
<th># of animals</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>ROA</th>
<th>Dose Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2M</td>
<td>[³H]ABBV-011, ABBV-011</td>
<td>23.75</td>
<td>IV bolus</td>
<td>once</td>
</tr>
</tbody>
</table>

Plasma time points: 6h, 24h, 48h, 72h post-dose
Samples analyzed by Radiolabeled Rat *In Vivo Metabolism Assay*

---

**Supplemental Table 2. Animal study information.**
Supplemental Table 3. Retention Time, Molecular Ions and Characteristic Fragment Ions of ABBV-011 and Radiolabeled Components in Rat Plasma.
Supplemental Figure 1. Radiolabeled LD19.10 structure.
Drug Metabolism and Disposition. Preclinical Characterization of Catabolic Pathways and Metabolism of ABBV-011, a Novel Calicheamicin-Based SEZ6 Targeting Antibody Drug Conjugate.

Daniel Ladror, Christine Gu, Vince Tong, Alexander Schammel, Julia Gavrilyuk, Tony Haight, Hetal Sarvaiya.

Supplemental Figure 2. ABBV-011 minor metabolite structures
Supplemental Figure 3A. M8b MS² Spectra
Supplemental Figure 3B. M1 MS² Spectra
Supplemental Figure 3C. M3 MS² Spectra