Complete characterization of KADCYLA using a new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer with electron-transfer/higher-energy collision dissociation (EThcD) fragmentation

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Abstract

Purpose: To demonstrate the capability of the new Thermo Scientific™ Orbitrap™ Excedion™ Pro BioPharma hybrid mass spectrometer for in-depth characterization of a Lysine-linked antibody drug conjugate.

Methods: A Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer was used for all experiments.

Results: Successfully characterized KADCYLA® at different levels, especially the Electron-transfer/higher-energy collision dissociation (EThcD) fragmentation provided conjugated sites information.

Introduction

Over the last decade, antibody-drug conjugates (ADC) have evolved into promising and efficient therapeutic agents for targeted chemotherapy in cancers, with 13 ADCs currently approved by the Food and Drug Administration (FDA), and more than 100 ADCs in clinical studies by October of 2024. ADCs are generated through the conjugation of monoclonal antibodies (mAbs) targeting specifically the tumor-associated antigens (TAAs) of the tumor cell with highly potent cytotoxic drug payloads via a cleavable or non-cleavable chemical linker. Here we demonstrated the comprehensive characterization of KADCYLA (ado-trastuzumab emtansine), a Lysine conjugated-ADC with a DAR distribution from 0-8, using a Thermo Scientific Vanquish UHPLC coupled to a new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer with electron-transfer/higher-energy collision dissociation (EThcD) fragmentation.

Materials and methods

Sample preparation

Native intact mass analysis: Commercially available KADCYLA was diluted to 10mg/mL using ddH_2O.

Subunit intact mass analysis: KADCYLA was diluted to 0.5mg/mL using 50mM Tris-HCl (pH=7.5) followed by IdeS digestion then DTT reduction.

Peptide mapping: KADCYLA was diluted to 1mg/mL using denaturing buffer (7M guanidine hydrochloride, 50mM Tris-HCl, pH=8.3), then reduced, alkylated and digested with trypsin or AspN respectively.

UHPLC Separation: A Thermo Scientific™ MAbPac™ SEC-1 column(P/N 088790), A Thermo Scientific™ MAbPac™ RP column (P/N 088648) and A Thermo Scientific™ Hypersil GOLD™ Peptide column (P/N 26002-152130) were used for separation.

Mass Spectrometry: A new Orbitrap Excedion Pro BioPharma mass spectrometer was used.

Data analysis

Data analysis was performed using Thermo Scientific™ BioPharma Finder™ software (version 5.3), Thermo Scientific™ Proteome Discoverer™ software (version 3.2) and Thermo Scientific™ Ardia™ (version 1.1) software.

Figure 1. The Schematic of KADCYLA[1].

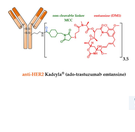
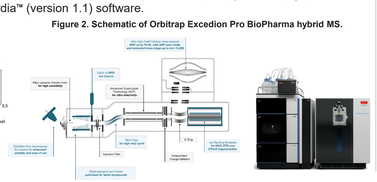


Figure 2. Schematic of Orbitrap Excedion Pro BioPharma hybrid MS.

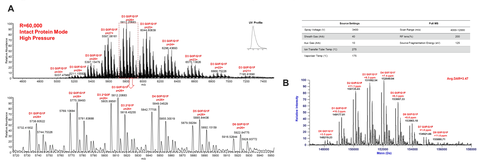


Results

Native intact MS analysis and DAR measurement

With conjugation at lysine residues, a distribution of DARs ranging from 0 to 8 drugs has been reported for KADCYLA[1], which indicates the molecule has high structure heterogeneity. To reduce the interference from adjacent MS signals, intact MS under native condition is essential for accurate measurement of molecular weight and DAR at intact ADC level. As shown in Figure3, the 0-8 DARs distribution can be observed. Low level of intact ADC D8 G0F/G1F (2.54% of top component) was also detected. The average DAR is 3.47, which is calculated by BioPharma Finder software automatically, in agreement to previous publication[1].

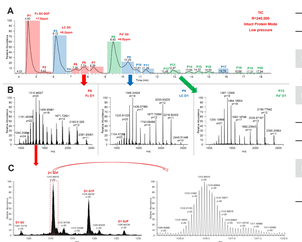
Figure 3. Native intact MS of KADCYLA. A, full MS raw spectra . B, deconvolution result.



Subunit analysis under denaturing condition

To further understand the heterogeneity of this biomolecule, analysis of subunits under denaturing conditions was conducted. 3 µg sample was loaded for analysis. Due to the highly structure heterogeneity of the molecule, 20 peaks for subunits carrying different payloads (D0 to D2) were chromatographically separated and successfully identified (Figure 4A-B). 240,000 resolution and intact protein mode (low pressure) were applied for subunit analysis data acquisition, provided baseline separation of isotopic peaks and highly sensitivity. Table 1 lists information of main components in each peak. Both N-glycoforms distribution in Fc region and payload distribution in subunits were detected.

Figure 4. Subunit analysis of KADCYLA under denaturing condition. A,RPLC separation profile. B, full MS spectra of peak6, peak9 and peak13. C, zooming-in view of peak6 full MS spectra.



Source Settings	
Spray Voltage (V)	3400
Sheath Gas (Arb)	40
Aux Gas (Arb)	10
Ion Transfer Tube Temp (°C)	275
Vaporizer Temp (°C)	175
Full MS	
Scan Range (*m/z*)	500-3000
RF lens(%)	60
Source Fragmentation Energy (eV)	0

Table 1. List of main components for the subunits.

Peak	Major species	Mono. Mass (Da)	Peak	Major species	Mono. Mass (Da)	Peak	Major species	Mono. Mass (Da)
Peak1	Fc D0 G0F	25216.623	**Peak10**	LC D1	24382.120	**Peak17**	LC D2	25338.622
	Fc D0 G0F	25217.762		Fd' D0	25364.761		Fd' D1	26322.233
Peak2			**Peak11**	LC D1	24381.175	**Peak18**	Fd' D2	27277.648
Peak3	LC D0	23424.636	**Peak12**	Fd' D1	26321.251		LC D2	25338.639
Peak4	LC D0	23425.744		Fc D2 G0F	27130.633	**Peak19**	Fd' D2	27277.650
Peak5	Fc D1 G0F	26174.065	**Peak13**	Fd' D1	26321.217	**Peak20**	Fd' D2	27277.684
	LC D0	23425.731	**Peak14**	Fd' D1	26321.254			
Peak6	Fc D1 G0F	26174.046		LC D2	25338.593			
Peak7	Fc D1 G0F	26174.044	**Peak15**	Fd' D1	26321.224			
Peak8	Fd' D0	25363.658		LC D2	25338.552			
Peak9	LC D1	24381.068	**Peak16**	Fd' D1	26321.224			
	Fd' D0	25363.698		LC D2	25338.596			

Peptide mapping and conjugation peptides identification

Peptide mapping is a widely used analytical method to characterize biopharmaceuticals, providing detailed information about sequence coverage, PTM identification, localization, relative quantification, sequence variants, and disulfide bonds. In this study, 10 µg of trypsin or AspN digested KADCYLA was loaded, achieving 100% sequence coverage with MS/MS identification for both enzyme-digested samples (Figure 5A-B). Using two different enzymes provides complementary peptide identification information. Both HCD and EThcD were employed for peptide fragmentation. HCD generates rich *b/y* ions for peptide sequence identification, but it also fragments the conjugated payload, making conjugation site localization challenging, especially for peptides with multiple lysines. For EThcD on the Orbitrap Excedion Pro BioPharma hybrid mass spectrometer, the generated *c/z* ions are transferred to the C-trap (purging event) and accelerated back into the ion routing multipole for supplemental higher-energy collisional activation, further improving *c/z* fragmentation efficiency and allowing generation of additional HCD type ions (*b/y*). This new design can get EThcD fragmentation with a short fluoranthene radical anions reaction time while keeping good fragmentation efficiency. EThcD on the Orbitrap Excedion Pro BioPharma hybrid mass spectrometer enables peptide fragmentation while keeping conjugated payloads intact, aiding in the localization of conjugation sites on peptides with multiple modification sites.

There are 46 potential conjugation sites in total on LC+HC of KADCYLA, including 44 lysines and two N-terminals, which means 92 conjugation sites for the intact molecule (Figure 6A). 43 out of 46 conjugation sites were identified in this molecule (Table 2). Figure 6B displayed the EThcD MS spectrum of peptide ADYEK(188)HK(190) from light chain, which contains two potential conjugation sites. EThcD fragmentation provided plenty of *c/z* ions for conjugation localization, As well as *y* ions. All these product ions can be used for peptide sequence identification and conjugation site confirmation. For this peptide, LC K188 was conjugated by payload.

The structure of DM1 contains a stereocenter (Figure 6C). MCC-DM1 conjugation imparts a stereocenter and causes peptides to elute as a doublet in C18 reversed-phase LC. Figure 6D showed the XIC and EThcD MS spectra of peptide DK(216)K(217)VEPK(221)SC from heavy chain, generated from AspN digestion. Stereocenter caused doublet peak pairs elution can be observed in a narrow time range. The high quality EThcD spectra provided sufficient information for conjugation sites localization for this peptide.

As reported before[2], EThcD fragmentation can provide *c*+57/*z*-57 ion pairs for aspartic acid isomerization confirmation. Figure 7 displayed the isomerization% of HC D283, the *c*+57/*z*-57 ion pairs were automatically labelled by software. Isomerization of other aspartic acids were also listed.

Other common modifications in biotherapeutic products, such as deamidation, succinimidation and oxidation were identified and relatively quantified (Figure 8). The most abundant N-glycoform is A2G0F (51.02%). The lysine truncation% is 98.21%.

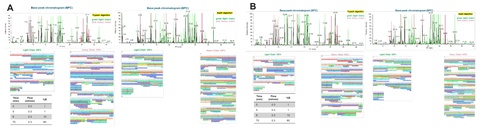


Figure 5. Peptide mapping sequence coverage using a 65min gradient. A, base peak chromatograms and sequence coverage map of trypsin and AspN digestion respectively, using HCD fragmentation (NCE=30%). B, base peak chromatograms and sequence coverage map of trypsin and AspN digestion respectively, using EThcD fragmentation (Supplemental Activation Energy=25%).

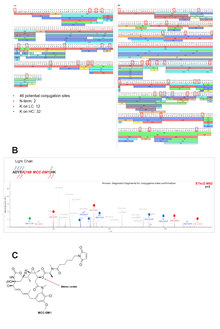


Table 2. Identified conjugation sites. "+", characteristic fragments were identified for location. "•", conjugation confirmed, lacking *b/y* ions coverage for conjugation sites. "-", conjugation is not identified.

Trypsin				
Chain	Site	HCD MS2	EThcD MS2	Sequence
LC	N-term	+	++	DIQMTQSPSSLSASVGDR
	30	+	++	ASQDVSTAVAWYQQKPGKAPK
	45	+	++	ASQDVNTAVAWYQQKPGKAPK
	N-term	+	++	DIQMTQSPSSLSASVGDRVTITCR
	103	+	++	SCTSTLTGSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK
	107	+	++	VEIKR
	126	+	++	TVAAPSVFIFPPSDEQLKSGTASVVCVLLNNFYPR
	145	+	++	DAVKWKIDNALQSGNSQESVTEQDSK
	169	+	++	DSTYSLSSTLTLSKADYEKHK
	189	+	++	VYACEVTHQGLSSPVTKSFNR
	199	+	++	GEC
HC	N-term	+	++	EVQLVESGGGLVQPGGSLR
	30	+	++	LSCAASGFNIKDTYIHWVR
	43	+	++	QAPGKGLEWVARIYPTNGYTR
	63	+	++	YADSVKGRFTISADTSKNTAYLQMNSLR
	76	+	++	FTGAHTSWATAYYLQMNSLR
	106	+	++	WGGDGFYAMKVWGQGTLVTVSSASTKGPSVFPLAPSSK
	136	+	++	GPSVFPLAPSSKSTSGGTAALGCLVK
	196	+	++	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
	206	+	++	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK
	213	+	++	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK
	216	+	++	VDKKV
	217	+	++	VEPKS
	221	+	++	SCDKTHTCPPCP
	225	+	++	DKTHTCPPCPAPELLGGPSVFLFPPKPK
	249	+	++	TKTCPPCPAPELLGGPSVFLFPPKPK
	251	+	++	TKTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
	277	+	++	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
	291	+	++	VKFNWYVDGVEVHNAKTKPR
	293	+	++	TVRR
	305	+	++	VVSVLTVLHQDWLNGKEYKK
	323	+	++	EYKCK
	325	+	++	CKVSNK
	327	+	++	VSNKALPAPIEK
	337	+	++	ALPAPIEKTISK
	341	+	++	TISKAK
	383	+	++	AKGQPR
	363	+	++	EEMTKNQVSLTCLVK
	375	+	++	LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
	399	+	++	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
	412	+	++	LTVDKSR
	417	+	++	LTYDKSR
	442	+	++	WQQGNVFSCSVMHEALHNHYTQKSLSLSPG

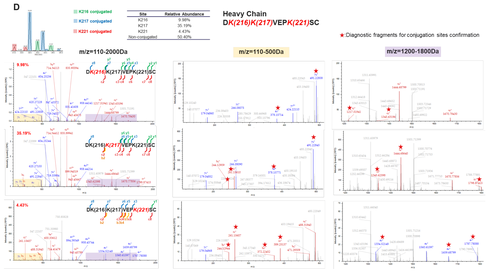
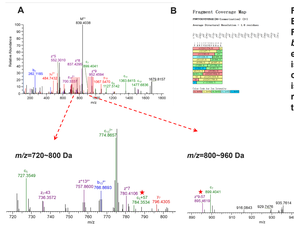


Figure 6. Conjugated sites identification. A, all conjugation sites in the protein sequence. B, EThcD MS2 spectrum of peptide ADYEK(188)HK(190) from light chain. C, The structure of MCC-DM1, the stereocenter was labelled with red arrow. D, XIC and EThcD MS2 spectra of peptide DK(216)K(217)VEPK(221)SC from heavy chain, which have three conjugation sites. The stereocenter caused peak pairs were eluted in 3 mins range, and the EThcD MS2 spectra provided confident evidence for conjugation sites confirmation.



C			
Residue #	Peptide Sequence	Isomerization % Avg	CV
HC D62	YADSVK	0.06	24.12%
HC D283	FNWYVDGVEVHNAK	0.18	4.64%
HC D404	TTPPVLDSDGSFFLYSK	0.18	13.60%

Figure 7. isomerization% of aspartic acid. A, EThcD MS2 spectrum for peptide FNWY*VD(283)*GVEVHNAK. EThcD can generate *b/y* and *c/z* ions for peptide identification. The expanded view displayed *c*+57/*z*-57 ions for isomerization confirmation. B, Fragment coverage map for this peptide. C, isomerization% for HC D62/D283/D404. All relative abundance% were calculated based on three replicate injections.

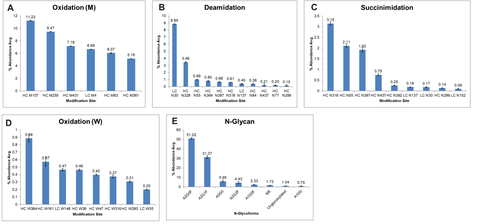


Figure 8. PTM report generated with Ardia. Trypsin digested KADCYLA sample, fragmented with EThcD was analyzed. A, Methionine oxidation. B, Asparagine deamidation. C, Asparagine succinimidation. D, Tryptophan oxidation. E, N-glycoforms on HC N300. All relative abundance% were calculated based on three replicate injections.

Conclusions

- In this work we demonstrated outstanding performance of a new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer for comprehensive characterization of KADCYLA, a lysine-linked antibody drug conjugate.
- High resolution and sensitivity benefits all applications, including native intact MS, subunit analysis and peptide mapping.
- EThcD fragmentation enables conjugation localization for peptide with multiple potential conjugation sites.
- All of the applications allow for DAR measurement, N-glycoforms, PTMs identification and confident detection conjugation site on peptides with multiple potential modification sites.

References

1. Pharmaceuticals 2020, 13, 245.
2. Anal. Chem. 2010, 82, 17, 7485–7491.

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