LOCALIZING THE CONJUGATION SITES OF CYSTEINE-CONJUGATED ANTIBODY DRUG CONJUGATES BY IMPROVED LC-MS SUBUNIT ANALYSIS FOR ADC POSITIONAL ISOMER IDENTIFICATION

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INTRODUCTION

Subunit analysis of antibody drug conjugates (ADCs) with LC-MS methods provides insightful structural information, such as conjugation isoforms and their modifications (e.g. oxidation, or water losses). This information is not readily accessible by either intact mass analysis or peptide mapping.

Cysteine proteases (e.g. IdeS or SpeB) are often used to cleave the hinge region to generate the LC, Fc/2 and Fd subunits because of their high specificity for mAb sequences. However, we discovered that the cleavage for ADCs was highly dependent upon the degree of conjugation, presumably due to steric hindrance.

we report here a study to address this cleavage efficiency issue by evaluating IdeS, SpecB, and Lys-C for the localization of conjugation sites and confirmation of positional isomers.

Figure 1. Experiments were conducted with the Acquity UPLC H-Class Bio and the Xevo G2S QTof MS or Xevo G2-XS QTof MS system (as shown).

METHODS

Sample Preparation

IdeS and SpeB (Genovis, Switzeland) and Lys-C (Roche, Indianapolis, IN) were used for enzymatic digestion of the ADCs. For IdeS or SpeB (100 units each) digestions, 50 µL of a cysteine-conjugated ADC sample (1 mg/mL) was incubated at 37 °C for 30 min before partial DTT reduction. Limited Lys-C digestion was achieved after optimization of the enzyme Lys-C to ADC (cysteine-conjugated) ratio and digestion time (30 min). Neat acetic acid was added to terminate all digestions. The digests were diluted to 0.5 µg/µL prior to LC-MS analysis. Multiple batches of cysteine-conjugated ADCs (with increasing drug load) were analyzed in this study.

Instrumentation

LC: Waters ACQUITY H-Class Bio
MS: Waters Xevo G2-S QTof and Xevo G2-XS QTof

Columns

ACQUITY UPLC Protein BEH C4 Column, 300Å, 1.7 µm, 2.1 mm X 50 mm (p/n: 186004495)

LC-MS (RP-LC/MS)

Mobile Phase:

A: Water
B: Acetonitrile/Isopropanol=60/40
C: 1% Formic Acid in waters
D: 1% TFA in Waters

Gradient Table

Time	Flow Rate	Composition A	Composition B	Composition C	Composition D	Curve
0.00	0.500	85.0	5.0	0.0	10.0	Initial
1.00	0.500	85.0	5.0	0.0	10.0	6
1.10	0.300	61.0	29.0	0.0	10.0	6
2.50	0.300	61.0	29.0	0.0	10.0	6
17.50	0.300	53.0	37.0	0.0	10.0	6
18.50	0.500	5.0	85.0	0.0	10.0	6
20.00	0.500	5.0	85.0	0.0	10.0	6
21.00	0.500	85.0	5.0	0.0	10.0	6
25.00	0.500	85.0	5.0	0.0	10.0	6
30.00	0.050	45.0	45.0	10.0	0.0	6

MS Conditions

Capillary: 3kV;
Sample Cone voltage: 150 v;
Source Temp: 500°C;
Desolvation Temp: 350 °C;
Desolvation Gas Flow: 800 L/h

RESULTS AND DISCUSSION

1. IdeS cleaves at the ...CDKTHTCPPPAPELLG/GPSVF... moiety below the hinge region

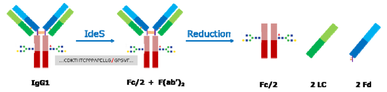

Figure 2. IdeS cleaves at the ...CDKTHTCPPPAPELLG/GPSVF... moeity below the hinge region, generates three 25k fragments, Fc/2, 2xLC and 2xFd. However, the tested cysteine-conjugated samples do not contain the targeted moiety. Therefore IdeS would not generate expected fragments. The experiment was not run.

2. SpeB cleaves at the ...CDKTHT/CPPPAPELLGGPSVF... moeity above the hinge region

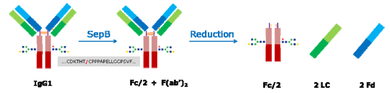

Figure 3. SpeB cleaves at the ...CDKTHTCPPPAPELLG/GPSVF... moeity below the hinge region, and generates three 25k fragments, Fc/2, 2xLC and 2xFd.

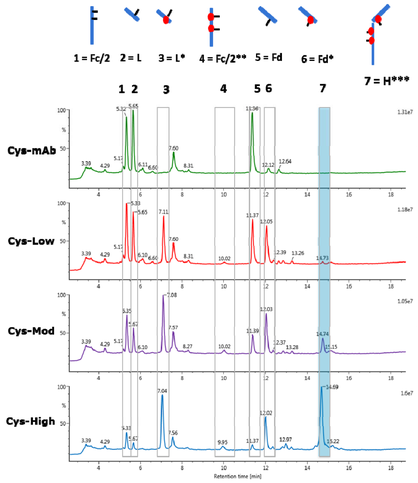

*Figure 4. LC MS TIC chromatograms comparison from the SpeB enzymatic digestion for the naked mAb and three different cysteine-conjugated ADC samples with increasing drug load. The 7 major fragmentations were confirmed by the MS spectra. The experiment indicates that the Sep B enzyme was capable of generating expected subunit fragments, however, it appeared that steric hindrance in the hinge region inhibits cleavage, resulting in H*** (+3 drugs) peak for higher drug loading ADCs as highlighted in blue in the figure.*

3. Limited Lys-C cleaves at the ...CDK/THTCPPPAPELLGGPSVF... moeity above the hinge region

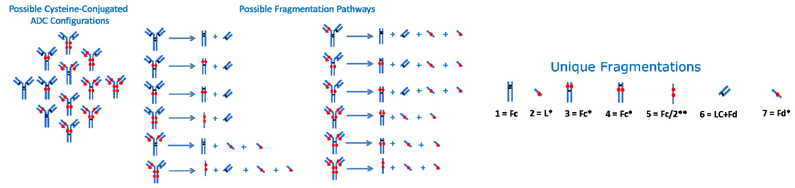

Figure 5. Limited Lys-C cleaves at the ...CDK/THTCPPPAPELLGGPSVF... moeity above the hinge region. Possible fragmentation pathways and unique fragments were shown in the figure.

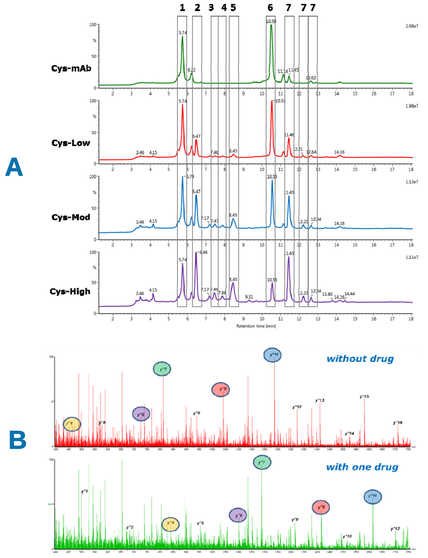

Figure 6. LC-MS TIC chromatograms comparison from the limited Lys-C enzymatic digestion for the naked mAb and three different cysteine-conjugated ADC samples with increasing drug load (figure 6A). The experiment indicates that the limited Lys-C enzymatic digestion protocol developed was much more effective to cleave the hinge region moiety than SpeB. This could may be because of the less steric hindrance for the cleavage in the limited Lys-C method compare to the SpeB approach.

The major fragmentations were confirmed by the MS spectra. Oxidation (spectra not shown) was observed from the Fc fragment (1 in figure 6A). Loss of water (spectra not shown) was observed from the LC+drug fragment (7 in figure 6A). The opening of the intra disulfide bonds (one or two pairs, spectra not shown) were observed for the Fc fragments as well as the LC+drug fragment. Positional isomers (3 and 4 in the figure 6A) can be confirmed by targeted MS/MS experiments after partial reduction. Figure 6B shows a pair of targeted MS/MS spectra (middle-down approach) from the Cys-High sample after reduction.

CONCLUSION

- **Limited Lys-C digestion protocol developed is more effective in the cleavage of the ADC hinge region moiety than the SpeB approach.**
- **Oxidation and loss of water were observed for the Fc and LC+Drug fragments.**
- **Positional isomers of Cysteine-conjugated ADCs can be confirmed by targeted MS/MS experiments by middle-down approach.**

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