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Short Communication

Characterization of antibody drug conjugate positional isomers at cysteine residues by peptide mapping LC–MS analysis

Marie-Claire Janin-Bussat[1], Marina Dillenbourg[1], Nathalie Corvaia, Alain Beck**, Christine Klinguer-Hamour*

Centre d'Immunologie Pierre Fabre, 5 Avenue Napoleon III BP 60497, 74164 Saint-Julien-en-Genevois, France

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ABSTRACT

Antibody-drug conjugates (ADCs) are becoming a major class of oncology therapeutics. Because ADCs combine the monoclonal antibody specificity with the high toxicity of a drug, they can selectively kill tumor cells while minimizing toxicity to normal cells. Most of the current ADCs in clinical trials are controlled, but heterogeneous mixtures of isomers and isoforms. Very few protocols on ADC characterization at the peptide level have been published to date. Here, we report on the improvement of an ADC peptide mapping protocol to characterize the drug-loaded peptides by LC–MS analysis. These methods were developed on brentuximab vedotin (Adcetris®), a commercial ADC with an average of four drugs linked to interchain cysteine residues of its antibody component. Because of the drug hydrophobicity, all the steps of this protocol including enzymatic digestion were improved to maintain the hydrophobic drug-loaded peptides in solution, allowing their unambiguous identification by LC–MS. For the first time, the payloads positional isomers observed by RP-HPLC after IdeS-digestion and reduction of the ADC were also characterized.

1. Introduction

ADCs combine the potency of cytotoxic drugs with the high specificity of a monoclonal antibody (mAb) and become increasingly important as new targeted therapies in oncology. Two ADCs, brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®) were recently approved by the US Food and Drug Administration [1], and more than 30 are currently being investigated in clinical trials [2]. The primary sites used for protein-directed conjugation are the amino groups of lysine residues or the sulfhydryl groups of the inter-chain cysteine residues, representing respectively one-third and two-third of ADCs in clinical trials [3]. Conjugates like brentuximab vedotin are formed through partial reduction of the antibody interchain disulfide bonds, followed by alkylation with a preformed drug-linker maleimide activated species. They result in conjugates with a distribution of drug loading from 0, 2, 4, 6 or 8 drugs incorporated per antibody and an average drug to antibody ratio (DAR) of ~4 drugs/mAb [4]. Conjugation of drugs to mAbs increases the structural complexity of the resulting molecule, which triggers the need for improved characterization methods [5] for analysis of DAR, drug distribution [6], size and charge variants, unconjugated drug, peptide mapping [7] and ADC biophysical properties [8]. Peptide mapping of ADC with hydrophobic drugs linked to its native cysteine residues by LC–MS analysis remains challenging due to the hydrophobicity of drug-loaded peptides [9]. This is especially true for peptides with two and more conjugated drugs.

Junutula et al. [10] described a site-specific conjugation of maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl-monomethyl-auristatin E (vc-MMAE) to an antibody through engineered cysteine substitutions at positions on light and heavy chains (ThiomAb-Drug Conjugate) resulting in only tree isoforms with 0, 1 or 2 drugs and no positional isomer. Tryptic peptide mapping with LC/MS detection of these conjugates identified four drug-conjugated peptides by a characteristic in-source fragmentation ion (m/z 718.5) that is observed in all mass spectra of molecules containing vc-MMAE. All peptides were identified as complete or partial tryptic cleavage fragments located around the

Abbreviations: ADC, antibody drug conjugates; DAR, drug to antibody ratio; Fc/2, constant fragment of IgG heavy chain; Fd, variable-CH1 fragment of IgG heavy chain; HC, heavy chain; IdeS, immunoglobulin degrading enzyme of *Streptococcus pyogenes*; IgG, immunoglobulin G; LC, light chain; mAb, monoclonal antibody; RT, room temperature; Rt, retention time; vc-MMAE, maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl-monomethyl-auristatin E.

* Corresponding author. Tel.: +33 4 50 35 35 83; fax: +33 4 50 35 35 90.
** Corresponding author. Tel.: +33 4 50 35 35 37.
E-mail addresses: alain.beck@pierre-fabre.com (A. Beck), christine.klinguer.hamour@pierre-fabre.com (C. Klinguer-Hamour).
[1] These authors contributed equally to this work.

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engineered cysteines. Surprisingly, to the best of our knowledge, no established protocol is currently published for endogenous mAbs cysteine residues-drug conjugates that yet represent two-thirds of ADCs in clinical trials. Here, we report on the improvement of an ADC peptide mapping protocol to characterize the payloads positional isomers using LC–MS platform.

2. Materials and methods

2.1. Reagents and materials

The ADC brentuximab vedotin (Adcetris©) was produced by Millenium Pharmaceuticals/Takeda (London, UK). Dithiothreitol (DTT), guanidine and Trizma-Base were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France), trifluoroacetic acid (TFA), acetic acid and formic acid from Fluka (Saint-Quentin-Fallavier, France), acetonitrile was obtained from Merck (Fontenay-sous-Bois, France), calcium chloride ($CaCl_2$) and isopropanol from Prolabo (Fontenay-sous-Bois, France), and Carlo Erba Reagenti (Peypin, France), respectively. RapiGest SF was purchased from Waters (Saint-Quentin-en-Yvelines, France), and Poros R1 resin from Applied Biosystems (Cergy-Pointoise, France). All the aqueous solutions were prepared using ultra-pure water.

2.2. ADC reduction and alkylation

Brentuximab vedotin was denatured by the addition of 6 M guanidine, 0.1 M Tris–Base, 2 mM EDTA, pH 8.0 buffer (v/v). Reduction was performed with 10 mM DTT for 45 min at 56 °C and alkylation with 25 mM iodoacetamide 30 min at RT in the dark. Acetic acid was then added to quench the reaction.

2.3. ADC IdeS digestion and reduction

Brentuximab vedotin was digested with immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (IdeS, Fabricator®) (Genovis Lund, Sweden) added at a ratio of 1 U/μg of ADC and incubated at 37 °C for 30 min. The sample was then treated as described in Section 2.2 without alkylation.

2.4. ADC IdeS fragments LC–MS analysis and isolation

Reversed phase separation was performed on an ultra-high performance liquid chromatography (UHPLC) system (Acquity UPLC, Waters, Milford, MA, USA) coupled to an electrospray mass spectrometer detector (LCT Premier ESI-TOF, Waters) for LC–MS analyses and to an UV detector (TUV, Waters) for peaks isolation. The reduced samples were directly injected on a PLRP-S column (2.1 mm × 150 mm, 8 μm, 1000 Å, Agilent) heated at 80 °C with a flow rate of 0.25 ml/min for analysis and a PLRP-S column (4.6 mm × 150 mm, 8 μm, 1000 Å) at a flow rate of 1.2 ml/min for isolation. Elution was performed with water as eluent A and acetonitrile as eluent B, both containing 0.05% TFA. The following elution gradient was applied, B was raised from 5% to 30% in 8 min and then from 30% to 50% in 40 min. The mass spectrometer was operated in positive mode and ions were scanned over a *m*/*z* range of 1000–3500. Fractions of interest were collected using UV detection at 280 nm and fragments recovered using Poros R1 resin (Applied Biosystems) following the manufacturer instructions.

2.5. Drug-loaded peptides mapping

Prior to Lys-C digestion, IdeS fractions corresponding to drug-loaded fragments were evaporated to dryness, reconstituted in 30 μl of digestion buffer (50 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.1) containing 0.1% of RapiGest surfactant (Waters), heated for 15 min at 80 °C and reduced with 20 mM DTT. Then, 10% acetonitrile was added to the sample. 1.5 μg of Lys-C was added to fractions, and digestion was performed for 3 h at 37 °C. A second reduction was performed by the addition of DTT to a final concentration of 35 mM followed by incubation for 45 min at 56 °C. After the second reduction, isopropanol was added to the sample to a final concentration of 40%. RapidGest was eliminated by acidification using a 10% TFA solution and incubation at 37 °C for 30 min, following the manufacturer instructions. LC–MS peptide mapping analysis was performed on an UHPLC system, with water as eluent A and acetonitrile as eluent B, both containing 0.1% formic acid. Digest was injected on an Acquity CSH C18 column (2.1 mm × 100 mm, 1.7 μm, 130 Å, Waters) heated at 40 °C. After an isocratic gradient at 0% B for 3 min, peptides were eluted from the column by increasing B to 60% in 60 min. MS analysis was performed on a LCT Premier ESI-TOF mass spectrometer (Waters) in W positive mode from *m*/*z* 150 to 2500 with voltages of 2 kV, 25 V and 5 V for capillary, sample cone and aperture 1, respectively. For in-source fragmentation, the aperture 1 voltage was increased to 40 V.

3. Results and discussion

3.1. Peptide mapping improvement

First, a classical protocol of mAb peptide mapping was applied with reduction and alkylation as described in Section 2.2. Exchange buffer was performed on Zeba™ Desalt Spin column (Thermo Scientific) following the manufacturer instructions. Lys-C digestion was performed as described in Section 2.5 excepted that there was no addition of acetonitrile. After digestion, RapiGest was eliminated as described in Section 2.5 and sample analyzed by LC–MS. The intensity of all peptide signals on the chromatogram was low due to the loss of loaded-HC and -LC. Then, the protocol was improved. The guanidine denaturation was replaced by heating without exchange of buffer before the Lys-C digestion to avoid the precipitation of loaded-LC and HC. To keep the loaded-peptides in solution after Lys-C digestion, 10% acetonitrile was added to the sample before digestion and 40% isopropanol after digestion. To avoid the presence of disulfide-bound peptides, a second reduction was introduced after digestion. With this improved protocol, all the loaded-peptides were identified on the LC–MS chromatogram.

3.2. Separation and identification of ADC drug-loaded fragments

The LC–MS analysis of reduced and alkylated brentuximab vedotin resulted in six major peaks (Fig. 1A) as reported by Lyon et al. [11], but with a better resolution for peaks 4 and 5 showing satellite peaks as reported by Le et al. [6]. The deconvoluted mass spectra of these six peaks show masses of 24,013 Da (Rt = 13.5 min) and 25,273 Da (Rt = 18.3 min) that matched with alkylated LC (MTheo = 24,013 Da) and LC + 1 payload (MTheo = 25,273 Da) and masses of 50,955 Da (Rt = 19.7 min), 52,215 Da (Rt = 22.2 min), 53,475 Da (Rt = 27.7 min) and 54,734 Da (Rt = 32.1 min) that matched with alkylated HC (MTheo = 50,957 Da), alkylated HC + 1 payload (MTheo = 52,216 Da), alkylated HC + 2 payloads (MTheo = 53,476 Da), HC + 3 payloads (MTheo = 54,735 Da). Two minor peaks were also observed and their deconvoluted mass spectra show masses of 52,216 Da and 53,475 Da that matched with alkylated HC + 1 payload and alkylated HC + 2 payloads, respectively. To summarize, alkylated HC + 1 payload and alkylated HC + 2 payloads fragments eluted at two different retention times (22.2 min and 23.0 min) and (26.9 min and 27.7 min), respectively, and could correspond to payloads positional isomers as described by Le et al. using the abundance of the different DAR species (HIC data), combined with capillary electrophoresis-based dissociation of the entire, unfractionated sample [6].