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Drug-to-antibody determination for an antibody-drug-conjugate utilizing cathepsin B digestion coupled with reversed-phase high-pressure liquid chromatography analysis

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ABSTRACT

Antibody drug conjugates or ADCs are currently being evaluated for their effectiveness as targeted chemotherapeutic agents across the pharmaceutical industry. Due to the complexity arising from the choice of antibody, drug and linker; analytical methods for release and stability testing are required to provide a detailed understanding of both the antibody and the drug during manufacturing and storage. The ADC analyzed in this work consists of a tubulysin drug analogue that is randomly conjugated to lysine residues in a human IgG1 antibody. The drug is attached to the lysine residue through a peptidic, hydrolytically stable, cathepsin B cleavable linker. The random lysine conjugation produces a heterogeneous mixture of conjugated species with a variable drug-to-antibody ratio (DAR), therefore, the average amount of drug attached to the antibody is a critical parameter that needs to be monitored. In this work we have developed a universal method for determining DAR in ADCs that employ a cathepsin B cleavable linker. The ADC is first cleaved at the hinge region and then mildly reduced prior to treatment with the cathepsin B enzyme to release the drug from the antibody fragments. This pre-treatment allows the cathepsin B enzyme unrestricted access to the cleavage sites and ensures optimal conditions for the cathepsin B to cleave all the drug from the ADC molecule. The cleaved drug is then separated from the protein components by reversed phase high performance liquid chromatography (RP-HPLC) and quantitated using UV absorbance. This method affords superior cleavage efficiency to other methods that only employ a cathepsin digestion step as confirmed by mass spectrometry analysis. This method was shown to be accurate and precise for the quantitation of the DAR for two different random lysine conjugated ADC molecules.

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1. Introduction

Antibody drug conjugates (ADCs) are a class of targeted chemotherapeutic agents gaining interest in the pharmaceutical industry. There are currently 84 open clinical trials being performed with ADCs in the United States registered with National Institutes of Health [1]. Two molecules, Kadcyla® (ado-trastuzumab emtansine) and Adcetris® (brentuximab vedotin), have already been approved demonstrating the proof of concept for ADC molecules [2].

ADCs are typically a hybrid of an ultra-potent cytotoxic small molecule drug linked to a large molecule antibody through either a cleavable or non-cleavable linker. The choice of antibody, drug and linker has to be carefully considered and is important to the clinical success of the molecule [3–6]. The antibody conjugation site is another critical consideration for the ADC chemistry and can be either site-specific or random. For random conjugation the linker is typically conjugated to the antibody at either lysines or thiols depending on its design [7,8]. Lysine conjugated ADCs result in a more heterogeneous population of drug attached to the antibody due to the higher proportion of lysines than thiols in the antibodies.

The amount of drug attached to the antibody is imperative for the safety and efficacy of ADCs. ADCs with suboptimal drug-to-antibody ratios (DARs) are prone to aggregation, poor solubility, and instability, which often lead to increased toxicity and/or inadequate efficacy in vivo [9–11]. Because of this, choosing the correct

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target DAR value is critical. Previous work indicated that an average DAR value of ~4 offers the best therapeutic index in ADCs [12]. Typical assays to establish the DAR value are UV, LC–MS and Hydrophobic Interaction Chromatography (HIC). Depending on the construct of the ADC, these assays can each have limitations. The method presented here overcomes common problems encountered in UV, LC–MS and HIC analysis such as the drug and antibody needing to have different lambda max values or potential changes to the absorptivity of the UV chromophore upon conjugation in UV analysis [13], the hydrophobic nature of the linker-drug and heterogeneity in the net charge of the protein due to modifications of the lysine residues that can affect the MS response [14] and lack of separation of the DAR species in HIC for lysine conjugated ADCs [15].

In this work, an approach for quantitation of the DAR for random lysine conjugated ADCs was developed. It focuses on the analysis of an antibody drug conjugate that employs a random lysine conjugation process for attachment of a tubulysin analogue to a human IgG1 antibody through a peptidic, hydrolytically stable cathepsin-B cleavable linker (Fig. 1). This method utilizes cleavage at the hinge region of the mAb and mild reduction prior to cathepsin B cleavage and overcomes the aforementioned limitations in other existing methods. The ADC is first treated with Immunoglobulin G-degrading enzyme of S. pyogenes (IdeS) to cleave the molecule into Fab′ and Fc fragments followed by 2-mercaptoethylamine treatment to mildly reduce to Fd′, Fc and Lc fragments (Fig. 1). By using IdeS instead of another protease such as Trypsin, the ADC is cleaved into smaller fragments but without creating a large number of peptides that would likely interfere with the quantitation of the drug. The use of 2-mercaptoethylamine (2-MEA) serves a dual purpose for reduction and also as an activator of the cathepsin B enzyme [16]. Following IdeS cleavage and 2-MEA reduction the sample is treated with cathepsin B enzyme to release the drug from the antibody fragments (Fig. 1). The method is free of interference from the protein and affords a universal method that can be used for ADCs that employ a cathepsin B cleavable linker irrespective of the conjugation chemistry.

As with any enzymatic treatment, the utilization of cathepsin-B for release of the drug could raise concerns about the completeness of the cleavage of the drug from the mAb. Quantitation of drug/mAb ratios in plasma samples using cathepsin-B cleavage has been reported for a thiol conjugated ADC using competitive ELISA [17]. The presence of cathepsin-B inhibitors in the plasma samples, however, did not allow for complete hydrolysis of the dipeptide linker and ultimately a loss of cleavage efficiency. Incomplete cleavage utilizing cathepsin B has also been demonstrated in other works [18–20]. In those publications the cathepsin B cleavage efficiency only reached approximately 73%.

To overcome the incomplete cleavage efficiency observed in other works, IdeS cleavage and 2-MEA reduction steps were performed prior to the cathepsin B treatment to allow the cathepsin B enzyme unrestricted access to the molecule. Due to the vast number of clinical targets and subsequent antibodies that can be used for ADC manufacturing; the IdeS cleavage/2-MEA reduction treatment ensures optimal conditions for the cathepsin B to cleave all the drug from the ADC molecule. The cleavage efficiency in the proposed method was evaluated and confirmed to be 100% complete utilizing mass spectrometry (intact mass analysis) to scan for masses corresponding to the Fd′, Fc and Lc fragments with drug attached. The cathepsin B cleaved drug was separated from the protein components by reversed phase high performance liquid chromatography (RP-HPLC) and monitored using the absorbance at 254-nm. The cathepsin-B enzyme does not distinguish between conjugated drug and free drug in solution; therefore, total DAR values are obtained. A universal method which overcomes the aforementioned limitations of other assays has been developed for analysis of DAR in ADC molecules irrespective of the conjugation site.

2. Experimental

2.1. Apparatus

Determination of the cleaved drug content in the formulated product was performed using a Waters 2695 separation module with a 2996 photodiode array detector (Waters Corporation, Milford, MA). The sample temperature was set to 6 °C and the column temperature was 65.0 °C. The flow rate was set to 0.25 mL/min using an absorbance of 254-nm for detection. The cleaved drug was separated on an Agilent Poroshell 300SB-C8 column, 5 μm, 2.1 × 75 mm by gradient elution using 0.125% TFA in water for mobile phase A and 0.0925% TFA in acetonitrile for mobile phase B. The gradient went from initial conditions of 90% mobile phase A/10% mobile phase B to 18% mobile phase A/82% mobile phase B over 18 min. The gradient was then returned to initial conditions over 2 min and held at initial conditions for 10 min prior to the subsequent sample analysis.

Efficiency of the cathepsin B cleavage for the two different enzyme ratios and confirmation of the UV peak identities by mass spectrometry were performed using a Thermo Scientific QExactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a Waters UPLC. In positive scanning mode, the source heater temperature was 425 °C and capillary temperature 350 °C. The spray voltage was set to 3500 eV and S-les Ref level 50 eV. The sheath gas and Auxiliary gas were tuned to 50 psi and 12 psi respectively. A mass range of 500–3500 Da, ion source CID of 60 eV and resolution 17.5 k were optimized to identify those subunit proteins.

2.2. Reagents and solvents

The 0.5 M EDTA and IdeS enzyme was purchased from Promega (Madison, WI). TFA was from Thermo Scientific Pierce (Grand Island, NY). The acetonitrile was from EMD Millipore (Billerica, MA). High purity deionized water purified by a Millipore Milli-Q system (Billerica, MA) with a resistivity of 18.2 MΩ cm was used for buffer and sample preparation. 4-Morpholineethanesulfonic acid (MES) hydrate, 1 M NaOH, sodium chloride, 2-mercaptoethylamine (2-MEA), cathepsin B enzyme and DMSO were from Sigma Aldrich (Saint Louis, MO). Polysorbate 80 (HX2) was from NOF America Corporation (White Plains, NY). The drug-linker standard was produced by Bristol-Myers Squibb (New Brunswick, NJ).

2.3. Method parameters

A drug-linker standard treated similar to the samples was used to construct the calibration curve for quantitation. The drug-linker was diluted with DMSO to yield a 5 mM (w/v) standard stock solution. The 5 mM stock solution was then diluted with DMSO to a 1 mM (v/v) standard working solution. The 1 mM standard working solution was further diluted with a 25 mM MES, 150 mM NaCl, 0.5 mM EDTA, 0.005% polysorbate 80, pH 5.5 buffer to a concentration of 50 μM (v/v). Since the standard is not expected to be cleaved by IdeS, the 50 μM standard solution was processed similarly to the samples with the exception of substituting an equal volume of the IdeS digestion buffer in place of the IdeS enzyme. The final concentration of the 50 μM standard after treatment is 20 μM. The IdeS enzyme used for the sample treatment was diluted per manufacturer's instructions to 50 unit/μL.

The ADC drug product samples were diluted to 1 mg/mL with IdeS dilution buffer (25 mM MES, 150 mM NaCl, 0.5 mM EDTA, pH 5.5) and the 50 unit/μL IdeS stock was then added at a ratio of 4 μL IdeS enzyme per 200 μL of sample which equates to 1 unit of IdeS

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