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Meeting Challenges for Analysis of Antibody Drug Conjugates

The complex structure of ADCs necessitates different analytical strategies than those for either small molecules or unconjugated monoclonal antibodies.

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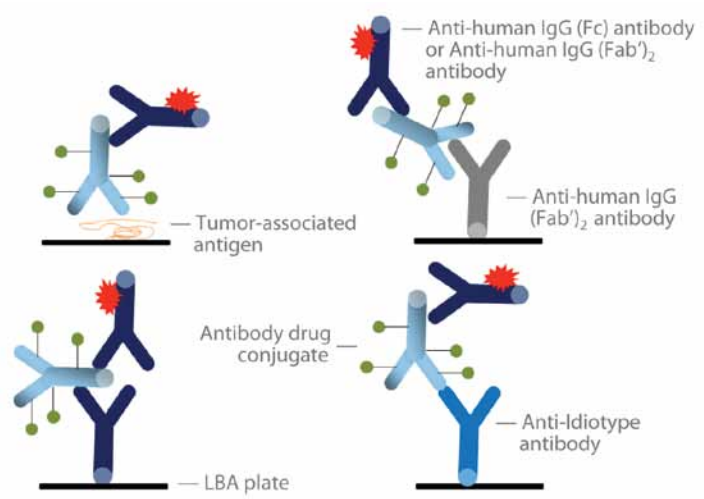
Chemotherapy is a mainstay of a standardized treatment regimen for cancer. However, the nonspecific targeting of healthy cells as well as tumor cells by cytotoxic small molecule drugs often results in intolerable side effects. These side effects compromise the efficacy of the treatment regimen and dramatically decrease the quality of life for cancer patients.

Antibody drug conjugates (ADCs) are a new class of chemotherapeutics which comprise monoclonal antibodies that selectively bind to tumor-associated antigens associated with a cytotoxic small-molecule payload. The payload is attached to the antibody using enzyme cleavable linkers. Much effort has been made to identify the highly tumor-specific monoclonal antibodies (mAbs), anti-cancer drugs with the maximum efficacy, and linkers that are stable in circulation but allow for rapid cleavage to release the cell-killing drugs following intracellular uptake of the ADCs. More than 30 targets have been investigated and more than 20 ADCs are now in various phases of clinical development, including Trastuzumab-DM1 (Roche), SGN-35 (Seattle Genetics), HuN901-DM1 (ImmunoGen), CR011-vcMMAE (Celidex Therapeutics), SAR3419 (Sanofi-Aventis), CMC-544 (Pfizer), and BIIB015 (Biogen Idec). Given the high complexity of ADCs resulting from the addition of the drug payload to already complex antibodies, the development and validation of analytical methods for ADC characterization, formulation analysis, and bioanalysis present significant challenges. A comprehensive review of bioanalytical assays for ADCs was published by Jean Philippe Stephan *et al.* In the current discussion, attention is focused on the development of bioanalytical assays for ADCs from a preclinical perspective.

Assay Formats for Pharmacokinetic Methods

The ADC is a heterogeneous mixture containing a cocktail of monoclonal antibodies with different drug payloads. Because of this heterogeneous nature, ligand binding assays (LBAs) are generally used for ADC bioanalysis. A variety of platforms are used including, colorimetric ELISA, CD-formatted Gyrolab, and electrochemiluminescence-based MSD. Total antibody assays can be used to quantify total antibody with or without the cytotoxic drug conjugated to it. Targeted tumor antigens, anti-idiotype monoclonal antibodies, anti-human IgG $(Fab')_2$, and anti-human IgG (Fc) antibodies

Figure 1. Example of total antibody assays.



can be used as capture reagents. Anti-human IgG $(Fab')_2$-HRP or anti-human IgG (Fc)-HRP, or biotinylated anti-human antibodies with streptavidin-HRP, are then used for detection (Figure 1).

To eliminate non-specific binding, monkey adsorbed anti-human IgG antibodies are commonly used for nonhuman primate studies. Depending on the type of linkers, the position of drug conjugation may be located on $(Fab')_2$, or the hinge region of the carrier antibodies. Increasing stoichiometry of drug conjugation may affect the binding of ADCs for capture, or detection reagents, and significantly affect the assay performance. Moreover, some assay formats are sensitive to the drug load even though the binding sites are not directly blocked. It has been reported that different assay formats yield different pharmacokinetic (PK) profiles and markedly affect the calculation of critical PK parameters such as clearance and drug exposure[3]. It appears that most of the assay formats are drug-load sensitive, but the generic human IgG assay using anti-human IgG (Fc) for capture and detection is an exception to this observation.

For conjugated antibody LBAs, the anti-cytotoxic drug antibodies are used as capture, or detection reagents paired with the capture, and detection reagents outlined above to measure the antibody which conjugates at least one drug (Figure 2).

Figure 2. Example of conjugated antibody assays.

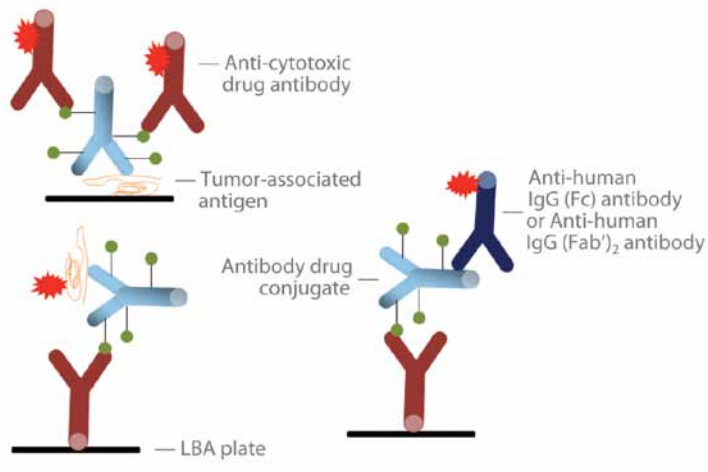
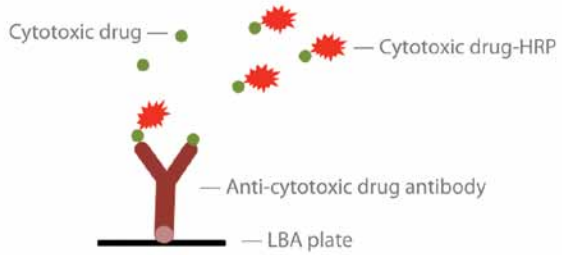


Figure 3. Competitive LBA for free drug.



The merit of conjugated antibody assays is their ability to quantify the possible progressive loss of drug from the ADCs in circulation. However, extra caution should be taken with the format using anti-drug antibodies for detection because the drug load of ADCs might change *in vivo*, compared with the ADC standard material used for the assays.

A successful design of an ADC is a combination of high drug-linker stability in circulation with efficient intratumoral cytotoxic drug release. Significant achievements have been made in the past years to develop more stable linkers[4]. However, the nonspecific release of drug from the carrier antibody in circulation is still a crucial factor in determining the half-life of ADCs. To measure the drug moieties that have been released from the carrier antibody (i.e., free drug), a competitive LBA format could be used with anti-drug antibodies coated for capture and a constant concentration of HRP-drug as the reporter (Figure 3).

Because the clearance of free drug released from ADC is much faster than the clearance of the ADC itself, the free drug might not be detectable. To solve this problem, one could measure the remaining drug that is bound to the ADC. This measurement can be achieved through the use of cathepsin B digestion to release the drug previously bound to the carrier antibody, followed by quantification of the free drug either via a competitive LBA assay or LC-MS.

Ideally, the assay methods for nonclinical PK bioanalysis should be developed during the early stages of ADC development and characterization. Evaluating the assays could be done with the recovery of enriched or purified drug antibody ratio (DAR) fractions compared with the average DAR standard to ensure that the different assay formats recover drug equally. If this analysis is not possible, detailed information of the ADC's mechanism of action, targeted tumor antigen, type of linker, drug antibody ratio, cytotoxic drug, etc., are necessary for the bioanalytical method design. In addition to LBA methods, hydrophilic interaction liquid chromatography (HILIC), HPLC, and LC-MS are being used to quantify ADCs. These methods, however, are beyond the scope of the current discussion and are reported elsewhere[1,3,4].

Immunogenicity of ADC

Although the same methods used for determining the immunogenicity of general therapeutic antibodies can be used to determine the immunogenicity of ADCs, further characterization of anti-ADC antibodies for the targeting antibody, the linker, and the drug components are required to address the specificity of positive samples. The complexity of the ADC structure raises additional questions not previously encountered in the analysis of monoclonal antibody therapeutics or small molecule drugs. For instance, does an antibody response against the linker or the cytotoxic drug affect ADC internalization? Alternatively, do only neutralizing antibodies against the complementarity determining region (CDR) of the targeting antibody reduce the efficacy of the ADC?

Matrix Selection

Antibodies are commonly recognized as stable; therefore, most bioanalytical assays for therapeutical monoclonal antibodies are established in serum. This blanket approach, however, is not appropriate for the bioanalysis of ADCs where the conjugation of small molecule drug to the antibody via a linker creates a molecule whose overall stability depends upon the least stable of the three components. To this end, plasma is suggested as being the preferred matrix used for PK, and immunogenicity sample analysis because the inhibition of the clotting cascade in plasma results in much less proteolysis than in serum. Moreover, protease inhibitors could be added during sample collection to further stabilize the ADCs in plasma.

Critical Reagents

The LBA has been the primary analysis platform used for ADC bioanalysis because of its many advantages, including the ability to measure the test article in matrix without further sample extraction, its high-throughput nature, the broad dynamic range and high sensitivity that can be achieved, and the requirement of minimal sample volume. However, the availability of critical reagents is a key component to the development of highly specific and sensitive LBA methods. Much time and effort is usually taken to create anti-idiotype antibodies against the carrier antibody or the cytotoxic drug. Because of the low immunogenic nature of cytotoxic drugs, conjugation with keyhole limpet hemocyanin (KLH) or an adjuvant may be required for antibody creation. The tight timelines for most IND-enabling studies necessitate early planning during the design of bioanalytical assays that factors in the timelines for generation of critical reagents. These critical reagents are either prepared in-house or subcontracted to third parties, and at least six months lead time is typically required. Antibody screening, assay format testing, and reagent purification are all steps that are part of the reagent generation process that will add even more time, cost, and risk to projects. In some instances, the anti-carrier antibodies and targeted antigens may be commercially available. In these cases, it is critical to secure sufficient quantities of the lot so that the reagent inventory can cover the entire study. Communication and establishing a good relationship with the reagent vendors are important and should be a key consideration. Should different reagent lots be used throughout the study, an appropriate way of assessing and bridging the different reagent lots must be established and implemented.

Conclusion

As ADC technology has become increasingly prevalent, it is imperative that new and reliable methods are developed to better characterize different ADCs both *in vitro* and *in vivo*. Because of the complexity of ADCs, many LBA formats are available for the same bioanalytical purpose. Therefore, caution has to be taken during assay development and validation to ensure that the selected method is the best fit for a given compound. The critical reagents are crucial factors for LBAs; therefore, sufficient lead time and effort must be factored into meeting project milestones.

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Yi Qun Xiao, PhD, is the Director of Immunology at MPI Research. Dr. Xiao joined the company in October, 2007. For nearly five years, he has played an important role in the expansion of immunotoxicity and large molecule capabilities in the Immunology Department. Dr. Xiao graduated from Beijing Medical University (Beijing University Health Sciences Center), China, and received his Master and Doctoral degrees of Pharmaceutical Sciences from Tohoku University, Japan. He was a research scientist and Director of New Product Development at Tianjin Lisheng Pharmaceutical Company in China, where he was employed for seven years. Dr. Xiao pursued his postdoctoral research at National Jewish Health Center. Prior to joining MPI Research, he was an instructor at the Division of Pulmonary Sciences and Critical Care Medicine at the University of Colorado Health Sciences Center. Dr. Xiao has extensive academic and industrial experience in immunology, cell and molecular biology noted by recognition in peer-reviewed publications such as *Cell*, the *Journal of Immunology* and the *Journal of Biological Chemistry*.

Andreea Halford, MS, MBA, Senior Manager of Ligand Binding Assay Development, joined MPI Research in July, 2008. She received her BS in Biochemistry from the University of Michigan, her MS in Pathology (immunology) from Case Western Reserve University and her MBA from Western Michigan University. Ms. Halford has extensive research experience in both biochemistry and immunology with emphasis on qRT-PCR, site-directed mutagenesis, cloning, protein expression and purification. She is also knowledgeable in characterization of protein structure and function via circular dichroism spectroscopy, plate-based binding assays, fluorescence plate-based co-immunoprecipitation assays, and fluorescence polarization assays. She has developed over 100 Good Laboratory Practice assays for PK and immunogenicity evaluations while at MPI Research. Ms. Halford has a wide range of biomarker and PK assay development experience including vast understanding of various ligand binding assay platforms including colorimetric ELISA, MSD, and Gyrolab®.

Roger Hayes, PhD, is Vice President and General Manager, Laboratory Sciences at MPI Research. Dr. Hayes has held numerous leadership positions in the global life sciences industry and academia, leading teams in the development of state-of-the-art bioanalytical and analytical techniques, including mass spectrometry, chromatography, and automation in bringing medical and chemical products to market. For nearly two decades, he led strategic and research initiatives for large pharmaceutical companies that included both GLP and non-GLP preclinical studies as well as clinical trials. At Merck Research Laboratories (2000-2010), Dr. Hayes directed the regulated bioanalysis group in support of preclinical and clinical safety trials and also advanced efficiencies for method development and validation. At Parke-Davis Pharmaceuticals (1993-2000), he managed the instrumentation support department, headed the bioanalytical groups for both safety and discovery research, and did cutting-edge work as the leader of the drug metabolism mass spectrometry group. Prior to that, Dr. Hayes spent several years at Procter and Gamble, where he developed new analytical technology and processes. His career is rooted in academia, beginning at the University of Adelaide in Australia, where he received his PhD and performed innovative research in gas phase ion chemistry. From there, he went to the University of Nebraska-Lincoln, where he served as an Assistant Director/Assistant Research Professor and continued his groundbreaking research, with an emphasis on advancements in the use of mass spectrometry. Most recently (2011), he served as President, bioanalytical operations, at Cetero Research where he focused on establishing overall corporate direction for bioanalytical and analytical services. Dr. Hayes has published extensively and has taught numerous aspects of LC/MS method development.

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