9th Annual European Antibody Congress, November 11–13, 2013, Geneva, Switzerland

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Keywords: antibody-drug conjugate, bispecific antibodies, protein scaffolds, antibody therapeutics, biosimilar antibodies

Abbreviations: ADC, antibody-drug conjugate; DAR, drug:antibody ratio; HER, human epidermal growth factor receptor; MS, mass spectrometry; PBMC, peripheral blood mononuclear cells; PK, pharmacokinetics

The annual European Antibody Congress (EAC) has traditionally been the key event for updates on critical scientific advances in the antibody field, and 2013 was no exception. Organized by Terrapinn, the well-attended meeting featured presentations on considerations for developing antibodies and antibody-like therapeutics, with separate tracks for antibody-drug conjugates, naked antibodies, and multispecific antibodies or protein scaffolds. The overall focus of the EAC was current approaches to enhance the functionality of therapeutic antibodies or other targeted proteins, with the ultimate goal being improvement of the safety and efficacy of the molecules as treatments for cancer, immune-mediated disorders and other diseases. Roundtable discussion sessions gave participants opportunities to engage in group discussions with industry leaders from companies such as Genmab, Glenmark Pharmaceuticals, MedImmune, Merrimack Pharmaceuticals, and Pierre Fabre. As the 2013 EAC was co-located with the World Biosimilar Congress, participants also received an update on European Medicines Agency guidelines and thoughts on the future direction and development of biosimilar antibodies in the European Union.

Note: The majority of the summaries in this report were based on PDFs of the presentations provided by speakers or input from the speakers. The authors wish to express their gratitude to speakers who could contribute to the report in this way. When a speaker could not contribute the PDF or input, detailed summaries were not prepared, although the names, affiliations and main points of all speakers were included in the report.

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Submitted: 01/17/2013; Accepted: 01/17/2013
http://dx.doi.org/10.4161/mabs.27903
(3) for HER targets, use of an active antibody capable of blocking oncogenic signaling can achieve multiple methods of cell-killing in the resulting ADC; and (4) appropriate patient selection is important.

Dr Lambert then described the development of IMGN289, which incorporates a novel EGFR-targeting antibody and proven ADC technology. The target is well-validated, and EGFR is known to be overexpressed in squamous cell carcinoma of the head and neck (SCCHN) and several subtypes of non-small cell lung cancer (NSCLC). The antibody component (J2898A; hulgG1) of IMGN289 was selected by screening panels of EGFR-binding antibodies for low impact on skin cells, rather than for high EGFR inhibition. It has similar anti-proliferative activity against tumor cells as cetuximab, but is less cytotoxic toward EGFR-dependent keratinocytes than cetuximab or panitumumab. The drug portion is DM1 (3–4 per IgG) conjugated to lysine of the mAb through a SMCC linker (thioether). IMGN289 displays high in vivo stability, with PK in mice following a single 10 mg/kg dose showing little apparent linker cleavage through thiol-maleimide exchange. In SCCHN and NSCLC models, the ADC showed increased anti-tumor activity, in a dose-dependent manner, compared with J2898A in tumors that are dependent (FaDu) or independent (NCI-H1703) of the EGFR pathway. A Phase 1 study (NCT01963715) of IMGN289 in adult patients with advanced EGFR-positive tumors was initiated in October 2013. Following the dose-finding phase in patients likely to have EGFR-positive tumors, an expansion phase will be limited to those with EGFR-positive solid tumors and will include patients with EGFR-resistant NSCLC, squamous cell NSCLC, SCCHN or any solid tumors.

Dr Lambert concluded by providing brief updates on the development of IMGN853, IMGN529 and IMGN901 (lorvotuzumab mertansine). IMGN853 is an ADC composed of an anti-folate receptor-α (FOLR1) antibody linked to DM4 via a sulfo-SPDB (disulfide) linker. The ADC is undergoing evaluation in a Phase 1 study (NCT01609556) of adults with ovarian cancer or other FOLR1-positive solid tumors. Initial data were presented at the 2013 American Society of Clinical Oncology annual meeting. Patients in the study had a mix of tumor types, treatment histories, target expression and drug doses received. An intolerable dose of 7.0 mg/kg was determined with once per 3-wk dosing (compared with 4.8 mg/kg for ado-trastuzumab emtansine), and activity was seen in three patients during dose escalation. IMGN529, an anti-CD37 ADC that incorporates the same linker and maytansinoid as ado-trastuzumab emtansine) is undergoing evaluation in a Phase 1 study (NCT01534715) of patients with relapsed or refractory non-Hodgkin lymphoma (NHL). The study is open to most prevalent types of NHL, and anti-tumor activity and side effects have been seen at doses that are lower than expected. IMGN901 targets CD56, which is found on cancers of neuroendocrine origin, e.g., SCLC, multiple myeloma. The ADC has been evaluated in ~200 patients as monotherapy and was generally well-tolerated, with some evidence of activity. A Phase 2 study (NCT01237678) to assess the safety and efficacy of IMGN901 in combination with carboplatin/etoposide in patients with extensive stage-disease small cell lung cancer was stopped in November 2013 on the recommendation of the data monitoring committee. Dr Lambert indicated that ImmunoGen is currently working to understand the findings from the study and to determine next steps.

Lutz Jermutus (MedImmune) discussed novel targets and antibody add-ons designed to enhance therapeutic utility. He started with a cautionary tale based on his work with glucose-dependent insulinotrop peptide receptor (GIPr), which is found on pancreatic β cells, as an example of a novel target. He noted that published studies suggested that inhibition of the binding of GIP, an endogenous hormonal factor, to GIPr should interfere with insulin response and might prevent obesity. Dr Jermutus then described the generation of antagonizing scFvs from phage and ribosome display libraries, which he mentioned were equally efficient. These were the first pharmacologically active antibody fragments that have been described against a class B GPCR. In particular, Gipg013 exhibited dose-dependent inhibition and was a competitive antagonist of GIPr. Fabs from Gipg013 were generated and a crystal structure of Gipg013 Fab in complex with the human GIPr extracellular domain (ECD) showed that the antibody binds to the N-terminal α-helix of the GIPr ECD and to residues around its glucagon receptor subfamily recognition fold. The Gipg013 binding site overlaps with the GIP binding site on GIPr. Preclinical studies, however, indicated that Gipg013 IgG had no statistically significant effect on the body weight of mice fed a high fat diet, even when dosed at 30 mg/kg, compared with the controls (irrelevant IgG, NaCl). Dr Jermutus noted that other biopharmaceutical companies (Amgen, Bayer) have also reported problems with replicating published study results, and this may at least partially explain why companies focus on well-validated target despite the competition. Regarding GPCRs specifically, Dr Jermutus noted that they are a druggable target class for mAb approaches, but requirements for agonist activity may make peptides a more feasible approach.

Dr Jermutus then discussed two approaches, phenotype-based and target-based, to drug discovery, but noted that the traditional target-based approach has the disadvantage illustrated in his example (i.e., target may not be important in disease). The phenotype-based approach, in which novel targets are found via screening for function, is more complex, but ensures that the link between biological response and the target is known before the process of drug discovery is started. Dr Jermutus described a study done at MedImmune that involved three membrane target discovery approaches. A proteomic approach, which used membrane protein enrichment followed by protein identification by mass spectrometry (MS), and two phenotypic antibody screening approaches, hybridoma-based and phage display-based, were used to identify targets on the MDA-MB-231 breast carcinoma cell line. A total of 40 cell surface markers were identified, with CD44 identified by all three approaches, and integrins α 3 and α 2, EGFR and CD73 identified by two of the approaches. Dr Jermutus indicated that an anti-CD73 antibody was generated and it demonstrated tumor inhibitory activity when administered at 10 mg/kg in a MDA-MB-231 xenograft model. In a second case study, he described the identification of P. aeruginosa exopolysaccharide by phenotypic screening and the
The design of mAb add-ons and replacements, e.g., bispecific antibodies and therapeutic enzymes, comprised the second main point of Dr. Jermutus’ presentation. Regarding bispecific antibodies, he first questioned whether these are living up to their promise. Technical and CMC issues have been addressed and over 50 different discovery approaches have been reported, but consistent clinical data has been generated only from CD3- or CD28-based bispecific molecules as well as those targeting related antigens, e.g., EGFR/HER3, HER2/HER3, IL-4/IL-13. In fact, substantial development hurdles remain, e.g., unexpected toxicity findings, separate engagement biomarkers. Dr. Jermutus suggested that bispecific development is simplified if both arms act on a single pathway, and infectious disease might represent a significant opportunity for bispecific antibody development. As an example, he discussed the development of various bispecific mAbs targeting P. aeruginosa virulence factor (PcrV) and persistence factor (PsI). The mAbs were composed of an anti-PcrV IgG backbone that incorporated anti-PsI scFv on the Fab, on the Fc or between the Fab and hinge region. All the multispecific antibodies retained anti-PcrV and anti-PsI functional activities in a single antibody molecule, but the molecule designed with the anti-PsI scFv between the Fab and hinge region afforded the highest percent protection in a P. aeruginosa acute pneumonia model (60% at 0.5 mg/kg dose to 100% at 5 mg/kg dose). He noted that a mAb mixture also afforded 100% protection at the 5 mg/kg dose, suggesting that antibody combination could be a credible alternative to bispecifics. On the topic of therapeutic enzymes, Dr. Jermutus noted that catalytic approaches can show superior acute and chronic efficacy over mAb approaches, but that they require further refinement. He briefly described results from a study of an amyloid-β degrading enzyme, neprilysin, which was fused to albumin to extend plasma half-life and engineered to confer increased degradation activity. In in vivo studies in rodents and monkeys, the engineered molecule depleted peripheral amyloid-β but did not affect central amyloid-β.5 The results are notable because they appear to disprove the amyloid-β “peripheral sink” hypothesis.

In the final keynote address, Andreas Plückthun (University of Zurich) discussed engineering protein ligands for very powerful biological responses. He first focused on the well-studied EGFR family (EGFR, ErbB2, -3, -4, which are also known as HER1 and HER2, -3, -4, respectively), which he indicated might not be as well-known as people think. He briefly reviewed the HER2 signaling paradigm and mechanisms proposed for how trastuzumab and pertuzumab interfere with signaling, but he indicated that resistance to the drugs, which develops at ~40 mo of treatment, suggests that the problem has not been solved yet and it is worth examining the biology anew. To do this, Prof. Plückthun questioned whether antibodies, either full-length or fragments, were necessary, and then introduced designed ankyrin repeat proteins (DARPins) as viable alternatives. The ankyrin repeat comprises 33 amino acids, with 2 antiparallel α-helices and a β-hairpin, and molecules with 2 to 23 repeats are found in nature. Designed versions can be linked in multimeric formats, do not aggregate, have no cysteines, can fold in the cytoplasm, and 100–200 mg/L can be made in shake flasks. Immunogenicity is expected to be low because the repeating nature of the protein limits the number of peptides (and thus the number of T cell epitopes), DARPins without T cell epitopes are selected, the molecules are resistant to aggregation (and thus T cell independent responses are unlikely) and they are small. Libraries of DARPins have been generated using ribosome, phage and yeast display technologies. Using a new recently constructed library, picomolar binders have been obtained after a single round of ribosome display, and, using an automated system, 96 independent selections can be done in parallel.

Prof. Plückthun then discussed how DARPins have been used to explore the biology of EGFR family members and to test whether cell-specific cytotoxicity can be induced. Initial exploration with monovalent DARPins was not successful; molecules with binding to the HER2 extracellular domain (ECD) with pM to nM affinity were generated, but these had no effect in a cell viability assay. After a long series of optimizations, the best constructs were found to be bi-epitopic anti-HER2 DARPins with short linkers. Prof. Plückthun presented data showing that these DARPins induce apoptosis via the intrinsic (mitochondrial) pathway in almost all HER2-overexpressing cell lines investigated, even though the DARPins are obviously extracellular reagents. In particular, DNA degradation, induction of caspase cascade, and the induction of apoptotic marker BIM were observed in cells treated with the DARPins (but not those treated with trastuzumab). The bispecific DARPins were also found to inhibit HER2/HER3 complex formation more efficiently than trastuzumab or pertuzumab in either the presence or absence of the HER3 ligand, heregulin.

To explore the in vivo activity, the DARPins can be modified to include a half-life extension module, e.g., human serum albumin, or branched PEG-40. Imaging studies in mice showed that PEGylated bispecific DARPins localize to tumors, and, administered at 20 mg/kg, the molecules showed anti-tumor activity. Prof. Plückthun indicated that the requirements for active constructs were examined next, as a first step to understand their mode of action. Changing the order of the domains, lengthening the linker, and using mixtures of homobivalent mixtures led to molecules with lower or even no activity. Since much greater avidity was observed for the bispecific constructs (e.g., 10 pM avidity on cells vs 90 pM for monovalent constructs), both domains must be engaged in binding. To better understand the exact interactions between the DARPins and HER2, crystal structures of complexes between a bispecific DARPin targeting ECD subdomains I and IV and the respective HER2 subdomains were obtained.6 An atomic model of the DARPin-linked HER2 was then built using all structural information: the extracellular domain DARPin complexes, the kinase, NMR of the transmembrane region. Exact modeling (distances) of all linkers and identification of all potentially flexible pieces was done. The results indicated that bispecific DARPins
crosslink two HER2 molecules and cause distortion such that signaling-competent dimers cannot be formed with any EGFR family member. He then showed data indicating that bispecific DARPinbs, but not trastuzumab, can inhibit phosphorylation of both HER2 and HER3. Prof. Plückthun noted that feedback loops are an important aspect of EGFR family signaling, and signaling from all receptors has to be completely cut off to stop growth and proliferation.

In concluding his presentation, Prof. Plückthun briefly described several other projects involving DARPinbs that are ongoing in his group, including the development of intracellular biosensors. The biosensor design, exemplified for ERK and pERK, was created by derivatizing a DARPin specific for phosphorylated ERK2 with a solvatochromatic merocyanine dye that shows increased fluorescence upon pERK binding.7 The sensor has been used to visualize differential activation of ERK in the nucleus, perinuclear regions, and especially the nucleoli of mouse embryo fibroblasts. In another approach, targeting of adenoviruses to cells via DARPin-based bispecific “adapters” was described. The DARPin “adapter” comprises two fused modules, one binding to the fiber knob of adenovirus serotype 5 and the other binding to various tumor markers. DARPin adapters have been generated that enable adenovirus serotype 5-mediated delivery of a transgene to cells via targeting to HER2, EGFR, or epithelial cell adhesion molecule.8 Prof. Plückthun’s final message to EAC participants was that protein engineering has a role to play in cancer therapy.

After the presentations, Dr Reichert conducted an interview with the three keynote speakers. All three were invited to comment on each of six questions, which were designed to give Dr Lambert, Dr Jermutus and Prof. Plückthun opportunities to expand on points made in their presentations. The questions were: (1) Given the wide variety of antibody formats, would you comment on whether you think that they all have potential as ADCs? For example, if target-independent toxicity due to binding of ADCs to cells expressing Fcy receptors is a concern, does it then make sense to use a non-Fc-containing targeting moiety? (2) Is the discovery of large (mAbs), medium (scaffolds) and small (peptides) binding moieties now a commodity and thus no longer the basis of a differentiated or innovative drug molecule? Is the world moving toward smaller binding moieties that offer CMC through chemical synthesis and possibly oral availability? (3) Can antibodies and other proteins can be used effectively intracellularly? (4) The value of reducing heterogeneity seems unquestionable, but, is it unlikely that true homogeneity of mAb products will ever be achieved. For ADCs in particular, has too much emphasis been placed on heterogeneity? (5) Amgen and Bayer have shown that the results from a large percentage of published “landmark” research studies cannot be replicated. As an industry, we look for great entry points for new therapeutic projects, but this requires substantial efforts within companies to reproduce published data and generate new targets internally. Which of the current approaches, e.g., genomics-based, phenotypic selection/screening-based, pooling of resources to repeat and validate published data, do you think are sustainable, and which are not worth the effort? (6) What are your thoughts on use of cellular or viral delivery of genes vs. delivery of proteins as therapeutics?

Following the afternoon break, Paul Polakis (Genentech) provided an update on two ADCs (DSTP3086S, DNIB0600A) of eight that Genentech entered into clinical studies during 2011–12. Both ADCs comprise a mAb conjugated to monomethyl auristatin E (MMAE) via a valine-citrulline linker; DSTP3086S targets six transmembrane epithelial antigen of the prostate (Steap1) and DNIB0600A targets NaPi3b, a multi-transmembrane, sodium-dependent phosphate transporter expressed in lung, ovary and thyroid cancers. Dr Polakis presented preclinical data showing in vivo efficacy of the anti-Steap1vMMAE construct (0.3, 3, and “5 mg/kg) in tumor xenografts generated with the LnCAP prostate cancer cell line, and a comparison of the efficacy of 5 mg/kg doses in prostate cancer explant models using LuCAP35v, LuCAP77, LuCAP70 and LuCAP96.1 cell lines. The reduction in tumor volumes compared with controls was greatest in the LuCAP35v and LuCAP70 models, and least in the LuCAP77 model. Dr Polakis mentioned that multi-drug resistance protein 4 is known to be highly expressed in LuCAP77. A Phase 1, dose-escalation study (NCT01283373) of DSTP3086S administered as a single agent by intravenous infusion to patients with metastatic castration-resistant prostate cancer was initiated in March 2011. In this study, doses (0.3 to 2.8 mg/kg) are administered every 21 d until disease progression or unacceptable toxicity. The preliminary PK profile supports the every 3 wk dose regimen; at the 2.8 mg/kg dose (n = 6), the half-life of the ADC was 5.7 d (SD = 1.8), while that of the total antibody (unmodified and drug-conjugated) was 9.6 d (SD = 1.16). Most adverse events (AEs) were Grade 1–2, and the majority were self-limiting and did not require intervention. Response evaluation criteria in solid tumors (RECIST) partial response was found to correlate with declines in circulating tumor cells and prostate-specific antigen in the 2.8 mg/kg cohort. A recommended Phase 2 dose (RP2D) has been identified, and DSTP3086S administered at the RP2D is being evaluated in an expansion cohort.

In discussing DNIB0600A, Dr Polakis mentioned that NaPi3b was identified by searching for targets with high expression in lung tissue irrespective of cancer specificity; the antigen was also found to be highly expressed in ovarian cancer. Dr Polakis also noted that a companion diagnostic prototype immunohistochemistry (IHC) assay is being developed for NaPi2b. Dr Polakis presented preclinical study data from administration of 1, 3, and 6 mg/kg of DNIB0600A to cynomolgus monkeys, with findings consistent with previous ADCs. The highest non-severely toxic dose was 3 mg/kg. DNIB0600A is currently undergoing evaluation in two Phase 1 studies and a Phase 2 study. Dr Polakis described results from the first-in-humans, dose-escalation study (NCT01363947), which was initiated in June 2011 and includes patients with non-squamous NSCLC or non-mucinous, platinum resistant ovarian cancer. DNIB0600A is administered at doses from 0.2 to 2.8 mg/kg administered every 21 d until disease progression or unacceptable toxicity. Most related AEs were Grade 1 or 2. The RP2D was identified as 2.4 mg/kg; fatigue, nausea, vomiting, headache and peripheral neuropathy are the most frequent related AEs at this dose. A
Alain Beck (Pierre Fabre) provided insights on the state of the art analytical methods for characterization of monoclonal antibodies, with a focus on ADCs. He started by describing a MS characterization flowchart going from top (intact IgG), to middle (large fragments derived from DTT, papain or IdeS treatment of the IgG), to bottom (peptides or glycopeptides derived from further treatment of fragments with DTT and enzymes), and analytical and structural methods used to assess mAb primary structure, higher order structures, aggregates, mAb/antigen complexes, as well as methods for quantification. Dr Beck then discussed the application of various MS methods that he and his colleagues have used to study several antibodies that are the focus of biosimilar mAb development, i.e., trastuzumab, rituximab and cetuximab. In particular, he noted the power of MS to identify correct antibody sequences and glycoforms, and establish a glycosylation profile for both Fab and Fc domains. This ability was critical to the discovery of a difference in the reported sequence of cetuximab’s light chain compared with that of the actual drug product. The approaches that he described are likely to be used extensively in non-clinical comparability exercises for biosimilar mAbs.

In discussing characterization of ADCs, Dr Beck first described three methods for conjugating drugs to antibodies: (1) lysine conjugation, as was done with trastuzumab emtansine; (2) conjugation through reduced internal disulfide bonds from cysteines native to the mAb, as was done with brentuximab vedotin; and (3) conjugation through engineered cysteines. He noted that the workflow for ADC characterization needs to take into account the DAR, load and distribution of drugs that result from the different conjugation methods. In particular, a panel of orthogonal methods (e.g., CE-SDS, RP-HPLC-MS, HIC, native MS) was recommended for ADC profiling. Dr Beck also discussed his work using an antibody-fluorophore conjugate (AFC) as a non-toxic model of an ADC. The AFC was based on the conjugation of dansyl sulfonamide ethyl amine (DSEA)-linker maleimide on interchain cysteines of trastuzumab used as a reference antibody. Dr Beck noted that the AFC modeled brentuximab vedotin and most ADCs in clinical studies. The AFC was first characterized by routine analytical methods (SEC, SDS-PAGE, CE-SDS, HIC, native MS), then IdeS digestion was performed, followed by reduction and analysis by liquid chromatography coupled to MS analysis. Dye loading and distribution on light chain and Fd fragments were calculated, as well as the average dye to antibody ratio for both monomeric and multimeric species. By analyzing the Fc fragment in the same run, full glyco-profiling and demonstration of the absence of additional conjugation was achieved. Dr Beck noted that there was a correlation between the number of conjugated payloads and the tendency for aggregation.

In concluding, Dr Beck emphasized that multiple liquid chromatography, electrophoresis and MS methods are used at all stages of mAbs discovery, preclinical and clinical development. The methods can aid, for example, in the selection of the best antibody-producing clones and provide full structural characterization of research leads and clinical candidates. Use of the methods are mandatory for comparability assays, formulation, process scale-up and transfer, and to define critical quality attributes in a quality-by-design approach. Routine and emerging methods can also help evaluate Fc-fusion proteins, biosimilar and biobetter antibodies, and more sophisticated and potent antibody derivatives, such as ADCs, multispecific antibodies, oligoclonal mixtures or high affinity protein scaffolds.

In the final presentation of the day, David Hilbert (Zyngenia) discussed monoclonal antibody therapeutics with up to five specificities, a functional enhancement that is achieved through fusion of target-specific peptides. The approach uses phage display technology to enable selection of target-binding peptides from structured random or domain-based libraries. The selected peptides can be fused to the N- and C- termini of both the heavy and light chains, affording the bivalent expression of up to four different peptides in modular mAb-based fusion proteins referred to as Zyodies. The mAb scaffold itself is not altered, and retains inherent affinity, binding specificity and valency, FeRn binding, and Fc receptor effector functions. Zyodies can be designed with 2–5 specificities and 4–10 valencies; yields of the molecules, which are produced in CHO cells, are comparable to that of canonical mAbs.

Dr Hilbert presented data for HER-per, a bispecific Zybody targeting two distinct HER2 epitopes and HER-per-egfr, a trispecific Zybody targeting the two HER2 epitopes and a single EGFR epitope. Trastuzumab is the scaffold mAb for the HER-per molecule, which binds to domain 2 and domain 4 of HER2. Dr Hilbert noted that the multi-epitope targeting of HER2 by HER-per drives internalization more efficiently (up to 40-fold) than mono-targeting with trastuzumab. When trastuzumab is armed with both additional HER2 specificity and an EGFR specificity, internalization is further enhanced and multiple signaling pathways are simultaneously downregulated. The tri-specific HER-per-egfr was shown to simultaneously downregulate EGFR and HER2 expression and intracellular signaling compared with trastuzumab or HER-egfr or HER-per Zyodies. Receptor downregulation was found to correlate with inhibition of HER2/EGFR-mediated signaling.

Dr Hilbert also discussed HER-1.1.3 trispecific Zyodies, which can be produced using either cetuximab or panitumumab as scaffold mAbs. The scaffold mAbs, which bind EGFR (also known as HER1), are modified to incorporate an additional HER1-binding domain and a HER3-binding domain, yielding trispecific Zyodies with bi-epitopic binding to HER1 and simultaneous binding to HER3. A series of HER-1.1.3 Zyodies were produced through a process of optimizing target binding, enhancement of in vivo stability, minimization of the immunogenic profile, and optimization of the spacer and linker. The Zyodies were produced in a CHO-based expression system with research batches (1–2 g) generated for in vitro and in vivo studies. The expression levels of stable isolates in non-optimized conditions in shake flasks varied depending on the
scaffold mAb (0.6 g/L for cetuximab-based Zybodies, 1.2 g/L for panitumumab-based Zybodies). Dr Hilbert presented data showing that HER-1.1.3 Zybodies have higher affinities to HER1 and lower affinities to HER3 compared with DL11F (MEHD7945A, Genentech), which is a HER1, HER3 dual targeting mAb undergoing evaluation in Phase 2 clinical studies of patients with colorectal or head and neck cancers. Unlike DL11F, Zybodies can bind to HER1 and HER3 independently. In particular, HER-1.1.3 Zybodies were found to inhibit HER1 and HER3 ligand binding, downregulate the receptors, and inhibit both HER1 and HER3-mediated signaling pathways. Lead Zybodies CET-13c and PAN-13c demonstrated significantly better efficacy compared with parental mAb and DL11F when dosed at 30 mg/kg in an in vivo model utilizing A431 human carcinoma cell line. Dr Hilbert concluded by noting that further differentiation will be achieved by binding of the current lead molecules and the product profile anticipates clinical differentiation by extending efficacy to broader tumor types as well as patient populations that have failed anti-HER1 therapy.

**Tuesday, November 12, 2013**

**Considerations For Developing Antibody and Antibody-Like Therapeutics**

Alain Beck

The plenary session of the EAC’s second day, chaired by Alain Beck (Centre d’Immunologie Pierre Fabre), was dedicated to discussion of the development of antibodies and antibodies-like therapeutics. Steffen Hartmann (Novartis) delivered a presentation on assessing antibody developability in the selection of optimal therapeutic antibody candidates. Antibody developability was evaluated based upon multiple parameters, including amino sequence liabilities, expression titer and purification yield, aggregation, stability, physicochemical profile, off-target binding, PK, half-life and immunogenicity. The starting point for antibody candidate selection was a large panel of antibodies with favorable biologic characteristics such as target antigen binding, in vitro potency and in vivo efficacy. Initial developability profiling was used to triage the antibody panel to -four candidates. More extensive developability profiling was then used to select a lead antibody for development.

Antibodies are susceptible to many different post-translational modifications (PTMs), including pyroglutamate formation, asparagine deamidation, aspartate isomerization, tryptophan and methionine oxidation, proline amidation and lysine glycation. The potential risk of PTMs on antibody developability varies from minimal to high, behooving case-by-case assessment. Major problems caused by PTMs that may be encountered include loss of potency, reduced safety, increased immunogenicity and altered PK. Other potential liabilities from antibody PTMs include reduced stability, problems in manufacturing, formulation and storage, plus the necessity of additional analytical methods. PTM profiling during antibody developability assessment included sequence-based prediction of potential PTMs and experimental evaluation, often under conditions chosen to accelerate their occurrence. It is sometimes possible to engineer the antibody sequence to remove the PTM site without perturbing binding affinity or biologic potency. Developability assessment also considered critical parameters such as aggregation by size exclusion chromatography, expression titer and purification yield, as well as other risk factors such as melting temperature, hydrophobicity and isoelectric point (pI).

Dr Hartmann described development of a traffic light ranking system where high, moderate and low risks were represented by red, yellow and green colors, respectively. High throughput formulation assessment was also included during candidate profiling. A case study was provided in which four Fab candidates were evaluated for an application requiring formulation at high concentration. The Fab with the best developability profile was selected based upon consideration of multiple parameters, e.g., pl, hydrophilicity, protein self-interaction, solubility, viscosity, purification experience. In addition to binding their cognate antigen, some antibodies show significant binding to other antigens. This so-called off-target binding poses multiple potential risks to antibody drug development, such as accelerated PK clearance, reduced efficacy and safety. Off-target binding was assessed by binding of the antibody candidates to chips with 384-arrayed proteins (Protagen). Binding of IgG to the neonatal receptor, FcRn, is important for maintaining the long serum half-life of antibodies. Binding of antibody candidates to FcRn from multiple species was evaluated by surface plasmon resonance. PK experiments in rats were included for in vivo fitness assessment of antibodies. Antibodies are potentially immunogenic in patients. Immunogenicity risk was assessed by proteomic identification of peptide sequences from antibody candidates that are processed and presented by MHC class II on antigen-presenting cells. In silico prediction was used to modify the antibody sequence to avoid MHC class II binding. Antibody variants were evaluated for biologic function, developability and proteomics to verify reduced presentation of antibody drug-derived peptides.

Pavel Strop (Rinat-Pfizer) gave a talk on the effect of the site of conjugation on the stability, PK, and toxicity of ADCs. Dr Strop first described the use of bacterial transglutaminase (TG) to make site-specific ADCs. A glutamine tag was introduced at 90 different sites in IgG, and 12 sites that had good biophysical properties and high conjugation yields were selected. These sites were located throughout the IgG, and therefore could be used to study how the conjugation site affects the properties of ADCs. Dr Strop also showed that the process is scalable, cleavable and non-cleavable linkers are compatible with TG conjugation, and that diverse cytotoxic compounds can be conjugated. Two selected sites (one at the C-terminus of the heavy chain, the second in the light chain) were conjugated with AcLys-VC-MMAD and compared with a mc-VC-MMAD conventional conjugate. The efficacy of the site-specific conjugates against the M1S1 target (drug-to-antibody ratio [DAR] 2) was comparable to the conventional conjugate (DAR4) in the BxPC3 mouse xenograft model; however, the site-specific conjugates were better tolerated. In the second part of the talk, Dr Strop showed data comparing the two site-specific conjugates in terms of stability and PK.
Data was presented showing that the site of conjugation can have an effect on the drug-linker stability. While no changes were observed in the TG linkage during the in vitro and in vivo stability studies, the ValCitz cleavage element appeared to be cleaved in circulation to some extent in the heavy chain conjugate. These findings appeared to be species-dependent, and the differences between the two sites were more pronounced in rats. The total antibody distribution was also changed based on the site of conjugation. The light chain conjugate showed comparable PK to wild type antibody, while the heavy chain conjugate had a faster distribution phase. These findings were also species-dependent and were more pronounced in rats. Dr. Strop concluded that the site of conjugation, the linker-payload, and the combination of the two, can modify properties of ADCs.

Tony Maschio (Maschio and Soames LLP) discussed how to understand and overcome intellectual property and patent law concerns when developing antibody therapeutics. He started with a recapitulation on the fundamentals of antibody patents. A first principle is that new and old technologies will generally result in broad and narrow patents, respectively. Sequence is not always the best way to define an antibody. Sequence-limited claims seldom protect against a dependent creation and re-optimization. Instead of sequence, a patent can rely on partial sequence (e.g., CDRs), target or epitope specificity, activity with respect to the target, the binding characteristics (affinity, competitive binding), clinical profile or method of making. There is no need to provide a structural definition of an antibody molecule and claims directed to “an antibody specific for protein x” are considered structurally definite. Methods for making antibodies are well known and most of the IgG antibodies share the same general structure.

Track A: Antibody-Drug Conjugates

Alexy A Lugovskoy

Following the plenary session, two tracks were available to the EAC participants. Track A was dedicated to discussion of ADCs. The session contained three talks on the technologies for ADC characterization and analysis and four case studies focusing on early-stage on ADC development. John Lambert (ImmunoGen), chaired the session. In his overview of the field, he noted the recent approval of trastuzumab emtansine (T-DM1) SEC buffer to improve resolution. Since no isopropyl alcohol effect was seen in the AUC experiments, Dr. Chen concluded that SEC “stickiness” was due to matrix interactions as opposed to aggregate formation. For charge variant analysis, Dr. Chen recommended the use of imaged capillary isoelectric focusing, which works well for both cysteine-conjugated and lysine-conjugated ADCs with the audience. She conceptually divided the techniques into two groups: (1) separation techniques that comprised chromatography and electrophoreases, and (2) detection techniques that comprised spectroscopy and MS. As hydrophobicity of the payload can cause aggregation, an approach that combined size exclusion chromatography (SEC), light scattering (LS) and analytical ultracentrifugation (AUC) was recommended. Dr. Chen suggested that optimized mobile phase may be needed to ensure SEC performance. For example, an isopropyl alcohol had to be added to trastuzumab emtansine (T-DM1) SEC buffer to improve resolution. Since no isopropyl alcohol effect was seen in the AUC experiments, Dr. Chen concluded that SEC “stickiness” was due to matrix interactions as opposed to aggregate formation. For charge variant analysis, Dr. Chen recommended the use of imaged capillary isoelectric focusing, which works well for both cysteine-conjugated and lysine-conjugated ADCs. In her opinion, this technique supplements capillary electrophoresis on SDS-PAGE (CE-SDS) data, which can be difficult to interpret due to chain dissociation. Dr. Chen described the use of UV-Vis spectroscopy to analyze protein-to-drug ratio based on the differences in the location of the maxima in absorption spectra for the antibody and the payload. This data can be corroborated by MS analysis. Hydrophobic exchange chromatography can also be useful for determining protein-to-drug ratio, particularly for cysteine-conjugated ADCs. Dr. Chen concluded by saying that she prefers a comprehensive approach to ADC analysis that incorporates both conventional analytical methods developed for monoclonal antibodies and specialized ones developed for ADCs.

Kevin Goosse (Quality Assistance) gave the perspective of a contract research organization (CRO) on the physico-chemical characterization of ADCs. After introducing Quality Assistance, a Belgium-based CRO, he focused on the variety of physico-chemical approaches useful for characterization of charge variants, DARs, size variants, and aggregation states of ADCs. For charge variant analysis, he recommended isoelectric focusing-based techniques with the specific emphasis on imaged...
capillary isoelectric focusing that had increased resolution and did not require an ADC immobilization step. Dr Goosse recommended a Bioanalyzer instrument for size variant analysis, and size exclusion chromatography coupled with multi-angle laser light scattering (HPLC-SEC-UV/RRI/MALLS) for aggregate analysis. The latter technique measures the amount of light scatter at each angle; the detected amount is proportional to molecular weight. For molecules that are sensitive to shear stress, Dr Goosse recommended the asymmetrical flow field flow fractionation coupled with multi-angle laser light scattering (A4F-UV/RRI/MALLS) technique, which, in his experience, while being less quantitative, is gentler because the ADCs do not interact with a stationary phase. Finally, for DAR analysis, he recommended hydrophobic interaction chromatography or a UV/Vis spectroscopic technique that relies on difference in the adsorption maxima between antibody and a payload.

Gulio Casi (Philochem AG) discussed curing cancer with non-internalizing ADCs. He presented his team’s work on non-internalizing ADCs that targeted molecular abnormalities of blood vessels at disease sites. Two case studies of antibody-like molecules that are VH/VL-CH4 anchored heterodimers directed at oncofetal fibronectin (F8) and oncofetal tenasin (F16) were discussed. Expression of these targets is restricted to placenta and endometrium in proliferative phase, while they are both prevalent on tumors. Philochem AG is pursuing F8- and F16-based ADCs, immunocytokine-drug conjugates and radioimmunoconjugates for use in oncology and chronic inflammation. The original biological proof-of-concept was achieved with F8-based immunophotodynamic therapy with a photoactivatable ADC prodrug. This molecule delivered curative outcomes in several subcutaneous mouse xenograft models. Next, Dr Casi discussed replacing the photosensitizer group with a cytotoxic drug, mertansine (DM1), that was linked through an engineered N-terminal cysteine. This ADC (F8-DM1) was active in mouse xenograft models despite not showing internalization. Finally, Dr Casi described an F8-interleukin 2 (F8-IL-2) fusion that showed a synergistic effect with F8-DM1 in a mouse model of acute myelogenous leukemia both as a combination or in a format of a single fusion protein (DM1-F8-IL-2).

David Rabuka (Redwood Bioscience) gave a presentation on SMARTag, a chemoenzymatic technology for generation of site-specifically conjugated ADCs. He remarked that conventional semi-specific conjugation technologies suffered from limitations of toxicity and variable potency due to significant heterogeneity, and that site-specific modification should be a preferred approach to ADCs going forward. The SMARTag technology utilizes aldehydes of formylglycine produced by formylglycine generating enzyme (FGE) acting on cysteine in a CxPxR motif. Redwood Bioscience has generated a Chinese hamster ovary (CHO) cell line that overexpresses FGE. An antibody that possesses CxPxR motif in the heavy chain, light chain or at the C-termini acquires an aldehyde when expressed in this cell line; a toxin can then be conjugated using aldehyde-directed Pictet-Spengler ligation chemistry. This technology offers excellent reproducibility of cysteine to formylglycine conversion, and the process is scalable up to 1000 L yielding over 1 g/L antibody titers. Redwood Biosciences has developed a library of cleavable and non-cleavable linkers that are stable in plasma for over 20 d. Dr Rabuka described preclinical validation of the technology in NCI-N87 xenograft model using re-engineered trastuzumab emtansine, which remained active irrespective of DM1 position placement. He remarked that Redwood Bioscience technology offers the opportunity to optimize PK and pharmaceutical properties of an ADC via differential payload placement.

Puja Sapra (Pfizer) discussed the innovations in ADC research at Pfizer oncology and provided a case study of novel auristatin-based ADCs directed at the Notch pathway. First, she described the development of a novel proprietary tubulin inhibitor platform at Pfizer. After screening a large library of cytotoxic compounds in vitro, the Pfizer team has prioritized 14 toxins. Of these, they selected vc0101 for use with a cleavable linker and mc3377 for use with non-cleavable linker. Both compounds were deemed to be safer (on the basis of rat toxicology study) and more active than competitor benchmarks. Interestingly, the cellular trafficking of these compounds was distinct: a cleavable linker one partitioned into endosomes, whereas non-cleavable linker went into lysosomes. Additionally, a vc0101 compound was not recognized by the ABCC1 (MPR1) transporter implicated in efflux of trastuzumab emtansine, thus it has a potential to overcome this mechanism of acquired resistance. Dr Sapra also presented a case study using these compounds in ADCs directed at a Notch protein that is active in tumor vasculature. Both toxins, when linked to either inhibitory or non-inhibitory anti-Notch antibodies, were readily internalized. All resulting molecules administered at 3 mg/kg yielded curative outcomes in a MDA-MB-468 orthotopic cell line xenograft model. No appreciable difference between different formats were detected. These findings were confirmed in a patient-derived xenograft model of non-small cell lung cancer. The lead molecule was then advanced into toxicity studies where it showed good safety profile in cynomolgus monkeys. Dr Sapra concluded by providing an overview of Pfizer ADC pipeline.

The final presentation of the session was delivered by Robert Rowlands (F-star) who described progress in converting an internalizing Fcab against HER2 into an ADC. In this specific case, the Fcab showed significant monotherapy activity in preclinical models (6 responders and 8 partial responders in 23 patient-derived xenograft models), and Dr Rowlands and colleagues thought to build on that by attaching a payload through a lysine-directed chemistry. He selected an RNA-polymerase II inhibitor amanitin, a bicyclic octapeptide isolated from the green deathcap mushroom Amanita phalloides that was discovered by Heidelberg Pharma GmbH. While no data on the constructed ADC was presented, Dr Rowlands promised to provide an update on the program at 2014 EAC.

Track B: Naked Monoclonal Antibodies

Thierry Wurch

Track B, which was chaired by William Finlay (Pfizer), addressed issues on engineering of naked mAbs and two examples...
of mAbs in clinical development. The first speaker in the session, Randall Brezski (Jansen R&D), presented a very interesting observation about the natural occurrence of antibody proteolytic breaks. Dr Brezski, who works in William Struhl’s group, noted that physiologically-relevant proteases either from the tumor microenvironment or secreted by microbes can cleave human IgGs within their hinge region, especially near the lower hinge/CH2 region [APELGGP region]. These breaks are often found in only one of the heavy chains (single-cleaved IgG or scIgG), which are strongly impair mAb effector functions, including both antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC). Conversely, mAbs belonging to the IgG2 isotype seem more resistant toward this proteolysis. Endogenous human IgG cleavage products of comparable size to scIgGs were detected by western blot analysis in synovial fluid from patients with rheumatoid arthritis and in breast carcinoma extracts. Nevertheless, their detection is problematic under physiological conditions due to the lack of good and specific detection antibodies, and because the structure of scIgGs is very similar to normal IgGs and their antigen-binding capacity is unaffected. Antibodies that specifically detect scIgGs, as opposed to normal intact IgGs, were generated by immunization of rabbits with hinge-specific peptides. These antibodies allowed the clear demonstration that scIgGs accumulate on the surface of opsonized tumor cells in the presence of proteases. Moreover, a single-cleaved version of anti-HER2 trastuzumab has lost all of the anti-tumor properties on the intact mAb in an in vivo mouse xenograft model. In order to avoid this loss of function, engineering of hIgG1 hinge region was performed to obtain protease-resistant (PR) mAbs still keeping the efficient immune-engaging properties. Several variants of PR mAbs were designed based on amino-acid exchange between the lower hinge of human IgG1 and IgG2 isotypes. Two variants were selected (PR AC1 and PR AC2) that exhibited protease resistance and strong ADCC, CDC and ADCP functions and serum half-life similar to intact IgG1.

Christian Frisch (ABD Serotec) discussed novel methods to generate anti-idiotypic antibodies (anti-ID mAbs) for the assessment of PK and immunogenicity (anti-drug antibodies) assays. In the past, human reference standards for immune-related assays could only be obtained from treated individuals (primate, humans), or by chimerization of mouse anti-ID mAbs. ABD Serotec has applied its human phage library technology (HuCAL) to generate fully human anti-ID mAbs. The entire selection process for human anti-ID Fabs takes 8 wk, followed by full IgG conversion. A first example showed the selection of anti-ID mAbs directed against the paratope of an anti-CD38 mAb. They exhibited potent capacity to inhibit binding of the anti-CD38 mAb to its cognate antigen. Specificity of paratope recognition was also assessed on a panel of unrelated antibodies constructed using the same framework regions. None of the anti-ID mAbs showed detectable binding to any of the unrelated framework matched Fabs. Finally, anti-ID mAbs could be applied as a detection reagent in a direct PK assay. The CD38 antigen was directly coated onto the ELISA plate. The sample containing the mAb to be dosed (e.g., the anti-CD38 mAb), as a pure mAb or within serum, was incubated and further detected using the anti-ID mAb (either directly labeled or revealed using a secondary mAb. In conclusion, such anti-ID mAbs may represent useful tools to evaluate anti-drug immune responses and powerful assay reagents. Their recombinant origin guarantees unlimited supply with limited batch-to-batch variations.

The next speaker, Thierry Wurch (Servier Research Institute), discussed his views on how to select best-in-class antibody hits. After a short summary of the current ways to generate antibody hits (i.e., conventional animal immunization, in vitro panning from human antibody fragment libraries), he presented three examples of antibody hit finding by applying a functional screen, and one example of antibody lead optimization by engineering of an original mAb candidate. The first example, which derives from a Servier partnership with MacroGenics Inc., involved selection of highly tumor-specific antibodies, generated by mouse immunization based on a screen by IHC. Once best tumor-selective mAb hits were selected, identification of the target antigen was performed by mass spectrometry (MS), which identified, in this example, B7-H3 as a target. B7-H3 corresponds to an immune checkpoint regulator that is overexpressed in various solid tumor types and often correlates with a poor outcome for the patient. Further antibody Fc engineering resulted in a highly cytotoxic and tumor-specific lead antibody that is being co-developed by Servier and MacroGenics and is currently in clinical Phase 1 study.

Dr Wurch also presented two mAb selection strategies based on the modulation of cellular functions that are critical for a tumor cell: proliferation and programmed cell death. Both approaches led to the identification of mAb hits with strong functional activity, both in vitro and in vivo mouse xenograft models. Target identification was performed either using a proteomic approach based on MS or using a lentiviral system to overexpress a series of 3000 cell surface proteins. Functional adhesion molecule-A (JAM-A) was identified as a target of interest to inhibit cell proliferation and Intercellular Junctional adhesion molecule-1 (ICAM-1) was revealed as a surface molecule that may trigger apoptosis. The latter mAb lead candidate, designated BI-505, is currently being sponsored by Bioinvent in a clinical Phase 2 study of patients with multiple myeloma.

Jerry Slootstra (Pepscan Therapeutics) then presented an interesting strategy to perform epitope mapping at a medium throughput level. Constrained peptides are used to functionally mimic a folded protein surface. Peptide-based protein mimicry is a useful method to develop antibodies against problematic proteins or to develop vaccines. Proteins that are composed of conformational or discontinuous sites cannot easily be mimicked by short linear peptides. The CLIPS (Chemically Linked Peptides onto Scaffolds) technology can be used to structurally fix linear peptides for experiments in solution and on solid phase arrays. Flexible designs to mimic local regions of complex proteins are possible. The epitope of several therapeutic antibodies was identified with high accuracy by using the CLIPS technology. Dr Slootstra presented a case study on epitope mapping of a series of anti-tumor necrosis factor (TNF) antibodies and comparative
analyses toward AZD9773 anti-TNF Fab. The study revealed that a number of different regions on TNF are involved in Fab binding, and probably three epitopes per monomer are responsible for most of the inhibition binding by AZD9773. The conclusion was that AZD9773, although being monoclonal by nature, can clearly bind to multiple epitopes on TNF and suggests a polyclonal behavior. He also showed that the CLIPS technology and fine epitope determination may also be applied for antibody affinity maturation.

After reconvening from the networking lunch, Nicolas Beltraminelli (Valneva) presented the strengths of the VIVAScreen™ technology platform dedicated to the identification and isolation of rare, therapeutic mAbs directly from human B cells. The technology has its foundations on the principle that humans naturally develop humoral immune responses, particularly the expression of antibodies, not only against exogenous but also against self-antigens. Even with all the advances made in antibody engineering, human-derived antibodies are still considered the best candidates for developing mAb therapeutics. Therefore, the VIVAScreen™ platform enables the isolation of human B-lymphocytes from peripheral blood using a high throughput, single-cell screening system based on a micro-array chip technology called ISAAC (ImmunoSpot Array Assay on a Chip). Access to this ISAAC technology was made possible through the acquisition in 2011 by Vivalis of the Japanese company Single Cell World Inc. The ISAAC technology allows rapid and precise mass screening of millions of B-lymphocytes. The VIVAScreen™ technology was made possible thanks to a privileged and quick access to thousands of samples of blood from healthy and diseased volunteers through an agreement with the French blood collection sites or "Etablissements Français du Sang." Following the screening of pools of B-lymphocytes for biological function, B-lymphocytes are captured individually in single wells using a micro-array system, one at a time. Candidate B-lymphocytes are identified using a known antigen of interest. Using a micro-pipetting technology, these individual cells can be isolated, individually, off the micro-array chip. After two weeks of culture, RNA of selected cells is retrieved by PCR and antibody genes cloned and produced as recombinant mAbs. The method also allows isolation of antigen-specific B cells present at low frequency (<2 x 10^-4) in peripheral blood of human donors. This approach was also applied successfully to select mAbs from B cells isolated from immunized animals such as mice and rabbits.

Jin-San Yoo (PharmAbcine) discussed one of the lead molecules in their portfolio. Tanimibumab (or TTAC-001) targets vascular endothelium growth factor receptor 2 (VEGFR2 or KDR) as an inhibitor of angiogenesis. Notably, the antibody is cross-reactive against VEGFR2 of human, mouse and cynomolgus species. It is a fully human IgG1 mAb with a binding affinity of about 15 nM to human VEGFR2, inhibiting VEGF binding and downstream intracellular signaling, but without internalization properties. Strong in vivo anti-tumor efficacy was observed as single agent in xenograft models of colon, breast, non-small cell lung and glioblastoma tumor models. Preclinical PK studies yielded a linear PK with dose-related area under the curve, PK/PD modeling allowed recommendation of the Phase 1 treatment schedule (5 to 20 mg/kg weekly dosage). A Phase 1 study was completed. From the preliminary data, taminibumab had no dose-limiting toxicity up to 24 mg/kg and no maximal tolerated dose was declared. Several patients yielded disease control. In contrast to other mAbs targeting the VEGFR pathway, taminibumab hasn’t cause any hemorrhage, bleeding and hypertension. Several biomarkers for biological activity were evaluated such as circulating levels of VEGF-A, soluble VEGFR2 and placenta growth factor. A Phase 2 study is in preparation in patients with recurrent glioblastoma multiforme and other potential indications are being explored.

The last speaker within this track was Michael Karl Bauer (Genmab), who presented an update on the development plan of daratumumab, Genmab’s lead candidate that has been co-developed with Janssen Biotech since the end of 2012. Daratumumab is a human anti-CD38 mAb with broad-spectrum killing activity. CD38 is a type II transmembrane glycoprotein that is highly expressed in hematological malignancies, including multiple myeloma (MM). Daratumumab has multiple mechanisms of action, including CDC, ADCC, antibody-dependent cellular phagocytosis (ADCP), apoptosis and modulation of CD38 enzymatic activity in vitro models. Synergistic activity was demonstrated in vitro in combination with standard MM therapies, including bortezomib and lenalidomide. Protective bone marrow stromal cells did not influence daratumumab-induced CDC and ADCC, suggesting that daratumumab may have activity in the bone marrow microenvironment in vivo. Indeed, significant daratumumab-mediated tumor growth inhibition was shown in MM mouse xenograft models. A Phase 1/2 clinical study is ongoing in patients with relapsed/refractory MM. Daratumumab has breakthrough therapy designation for treatment of patients with multiple myeloma who have received at least three prior lines of therapy including a proteasome inhibitor and an immunomodulatory agent. Daratumumab was undergoing evaluation in two Phase 1/2 and one Phase 2 study of multiple myeloma patients. The Phase 2 study (NCT01985126), which is in the breakthrough therapy designation indication, was initiated in October 2013 and has a primary completion date of October 2016.
(ibrutinomab tiuxetan, tositumomab I-131), one bispecific antibody (catumaxomab) and three Fab (abiximab, ranibizumab, certolizumab pegol). The biopharmaceutical industry has recently devoted substantial resources to development of non-canonical antibodies, however, and more of these are now entering clinical studies. Of the current clinical pipeline of ~360 antibody therapeutics, -23% are non-canonical molecules.

Dr Reichert then discussed the antibodies in clinical studies specifically for cancer indications and those studied for immune-mediated disorders, which together comprise ~75% of the clinical pipeline. Not surprisingly, non-canonical molecules are more prevalent among those studied for cancer indications compared with those studied for immune-mediated disorders. Non-canonical molecules, including 35 ADCs, comprise ~34% of the total anti-cancer pipeline, but only ~9% of the pipeline of antibodies for immune-mediated disorders. She remarked that examination of the pipeline overall reveals diversity in the antigenic targets of the antibodies, but clustering around certain targets can be seen. For example, the cancer pipeline includes multiple mAbs that target EGFR, HER3, PD1 or PD-L1, IGF-1R, CD19, HER2 and VEGF, while the pipeline of antibodies for immune-mediated disorders includes multiple mAbs that target IL-6, IFN, GM-CSF, IL-13, IL-17, and integrins. Dr Reichert concluded with a brief discussion of the biopharmaceutical industry’s preclinical pipeline, which is estimated at over 200 molecules and appears sufficient to maintain the current average rate of antibodies entering first-in-human studies (~60 molecules per year).

Guy Hermans (Ablynx) discussed the development of Nanobodies as therapeutic agents. Nanobodies are small, targeted biologics derived from heavy chain-only antibodies found in animals of the Camelidae family. Properties such as half-life, affinity, and valency can be tailored for specific uses, and the molecules can be used as the antibody component of an ADC or radioimmunotherapy. Ablynx currently has seven Nanobodies in the clinic, three at Phase 2 and four at Phase 1, that are sponsored by Ablynx or its partners. Dr Hermans then presented data for five Nanobodies that have been evaluated in clinical studies: (1) caplacizumab (ALX-0681), a bivalent, monospecific Nanobody with no half-life extension that targets von Willebrand factor; (2) ALX-0141, a trivalent, bispecific Nanobody, with albumin-binding ability for half-life extension, that targets RANKL; (3) ALX-0061, a bivalent, bispecific Nanobody, with albumin-binding ability for half-life extension, that targets IL6R; (4) TAS266, a tetravalent, monospecific Nanobody with no half-life extension that targets DR5; and (5) ALX-0171, a trivalent monospecific Nanobody with no half-life extension that targets respiratory syncytial virus (RSV). Dr Hermans emphasized that, in Phase 1 studies, ALX-0171 is administered directly to the lung via aerosol. In concluding, he remarked that Nanobodies are extremely modular, which allows control over compound valency, specificity, half-life and effector function, and that, as illustrated by the examples he presented, Nanobodies reflecting highly variable designs have been upscaled successfully and proved to be both safe and efficacious in clinical studies.

Alexey Lugovskoy (Merrimack) discussed the development of multispecific antibody molecules for the treatment of cancer, with a focus on MM-111, an anti-ErbB2/B3 bispecific antibody, and MM-141, a tetravalent bispecific antibody antagonist of IGF-1R and ErbB3. He began by pointing out that while genetic mutations may drive oncogenesis, ligand-driven mechanisms play an important role in metastasis and in resistance to therapy, and system dynamics determine outcomes. Merrimack’s network biology approach to drug discovery utilizes models trained on vast data sets to prioritize targets, therapeutic modalities, design parameters, clinical indications, drug combinations and companion diagnostics. Predictions are confirmed in preclinical models and by mining clinical data. A critical network identification screen involving 54 cell lines that were stimulated with 60 ligands was used to reveal key networks activated in cancer. The results have emphasized the importance of survival pathway activation by growth factors such as heregulin, which binds to the ErbB3 receptor.

Dr Lugovskoy then described the development of the anti-ErbB2/B3 molecule MM-111, which is a single polypeptide fusion protein composed of two human scFv antibodies linked to modified human serum albumin.33 The anti-ErbB2 docking arm of the molecule binds with K<sub>D</sub> of 0.3 nM and the anti-ErbB3 inhibitory arm binds with K<sub>D</sub> of 16 nM. MM-111 forms a trimeric complex with ErbB2 and ErbB3, and inhibits ErbB3 activation in ErbB2-high tumors more effectively than anti-ErbB3 IgG. Preclinical studies in a BT-474-M3 breast cancer xenografts model indicated that heregulin inhibition of paclitaxel can be rescued by MM-111 in vivo, and studies in a NCI-N87 ErbB2 high, heregulin-secreting gastric xenografts model indicated that MM-111 synergistically combines with trastuzumab to block heregulin-driven paclitaxel resistance. MM-111 has been evaluated in Phase 1 studies in patients with ErbB2-positive solid tumors, and a Phase 2 study (NCT01774851) of MM-111 and paclitaxel with or without trastuzumab in patients with ErbB2-expressing carcinomas of the distal esophagus, gastrosophageal junction and stomach, but who have failed front line metastatic or locally advanced therapy, is ongoing. MM-111 has received orphan drug designation by the FDA for development in the treatment of advanced gastric and esophageal cancers.

Dr Lugovskoy’s final topic was the development of MM-141, which is composed of an anti-IGF-1R IgG1 antibody (K<sub>D</sub> = 0.3 nM) genetically fused with an anti-ErbB3 antibody fragment (K<sub>D</sub> = 0.9 nM). He noted that IGF-1R blockade with antibodies is ineffective as autocrine heregulin compensates for IGF-1 receptor blockade, and ErbB3 and AKT are activated upon IGF-1R inhibition. MM-141 can inhibit PI3K/AKT/mTOR by blocking growth-factor induced signaling via both IGF-1R and ErbB3, and by causing degradation of receptor complexes of that include either of the two targets. Dr Lugovskoy showed preclinical data showing that MM-141 enhances the biological effect of receptor inhibition in vivo as a monotherapy and in combination with everolimus, gemcitabine or docetaxel, through blockade of IGF-1R and ErbB3 signaling and prevention of PI3K/AKT/mTOR network adaptation.34 MM-141 is undergoing evaluation
in a Phase 1 study (NCT01733004) of patients with advanced solid tumors.

Bo Kara (Fujifilm Diosynth Biotechnologies) discussed overcoming manufacturing challenges and streamlining the development of ADCs. He emphasized the challenges presented by the antibody in relation to the conjugation of the drug, ADC formulation, and the cost of goods. In particular, he noted that while manufacturability of antibodies is generally well-understood, ADCs pose additional challenges regarding the site of conjugation and the effects on stability and PK, extent and control of heterogeneity, optimization and characterization of the drug:antibody ratio (DAR), and the analytics specific to ADCs. Formulation of ADCs can also present problems because conjugation can affect the stability, solubility, or other properties of the molecule. For example, many small molecule drugs are relatively hydrophobic and thus may cause hydrophobicity-driven aggregation. Dr Kara discussed an example of an integrated approach designed to streamline development of ADCs using a “holistic” process to achieve final cost-of-goods targets for a flexible but fast track “as possible” program. In this program, three antibody variants and two conjugation chemistry options were evaluated, a formulation was developed, and scale-up and manufacturing of 1 kg of ADC was completed within ~1 y.

Dr Kara emphasized the importance of a process design that includes risk assessments based on ICH guidelines and evaluation of the effects of each individual parameter on the critical quality attributes. For ADCs, these parameters include partial reduction and/or modifications, with pH, ionic strength and/or buffering, the linker, redundant equivalents and kinetics, protein concentration, agitation and hold time stability to be considered, as well as aspects of conjugation (e.g., effects of co-solvent, stabilizing excipients, drug excess, quenching). As an example, Dr Kara discussed the importance of understanding reduction kinetics because the ratio of the antibody to the disulfide reducing agent tris(2-carboxyethyl)phosphine (TCEP) and pH can affect the DAR achieved. He concluded by briefly reviewing relevant points on setting specifications and testing of the mAb, drug/linker and ADC as outlined in the October 2012 presentation by US Food and Drug Administration’s Marjorie Shapiro, Office of Biotechnology Products, and Sarah Pope Mikinski, Office of New Drug Quality Assessment (www.fda.gov/downloads/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/UCM341177.pdf)

**Track A: Bispecifics, Multispecifics and Protein Scaffolds**

Steven Coats

After the morning break, participants could choose between two tracks. Track A, chaired by Steven Coats (MedImmune) focused on engineering novel formats, manufacturing bispecifics and development of next-generation antibody-related products.

Daniel Steiner (Molecular Partners) delivered a presentation on novel therapeutics concepts based on multi-functional DARPinCs. DARPinCs are small molecular weight, highly abundant binding proteins that are based on ankyrin-repeat proteins found in nature. Their small size and highly potent binding properties, combined with high stability and solubility, make them ideal starting points to develop multispecific DARPinCs. Three projects that use DARPinCs as either a multispecific approach to target two pathways (VEGF/HGF) or to bind paratopically to two epitopes on a single antigen (HER2, IgE) were described. MP0250 is a first-in-class anti-VEGF and anti-HGF antagonist to target both angiogenesis and cell proliferation. MP0250 demonstrated potent inhibition of both VEGF driven endothelial cell proliferation, as well as HGF mediated cell scattering and migration. This novel DARPinC also inhibited the growth of both glioblastoma tumor xenografts and renal cancer orthotopic tumor models. The first human studies of MP0250 are planned for the first half of 2014.

The second DARPinC that Dr Steiner described was a second-generation HER2 targeting agent that linked together two monovalent DARPinCs to bind two unique epitopes on HER2.

**Paul Parren** (Genmab) presented a case study in overcoming challenges with the reliable production of bispecifics. The insight that bispecific antibodies can be generated by Fab-arm exchange was obtained from their studies of IgG4 antibody. This knowledge was exploited to build the DuoBody platform, which enables the generation of bispecific IgG1 antibodies. DuoBody molecules specifically retain a wild-type IgG1 hinge to ensure stability in vivo. By scanning the CH3 interface, matched mutations were identified that allowed the dissociation...
PK. In silico immunogenicity prediction algorithms demonstrate pair to a bispecific antibody with normal effector function and heterodimer formation. The overall structure and glycosylation pairs, new heterodimeric interfaces can be built that facilitate receptors interfaces to mimic the natural association of T cell surface BEAT (Bispecific Engagement by Antibodies based on the T is a “Plug & Play” approach to bispecific antibodies. The first half of 2014.

T regulatory cells. Clinical studies are anticipated to start during without the toxicity associated with binding CD25 and activating activation, which results in NK cell mediated tumor cell killing IL-2. The IL-2 variant stimulates immune cell proliferation and fusion proteins have superior properties compared with wild type IL-2. Overall, these results demonstrate that the antibody/IL-2 variant fusion proteins have superior properties compared with wild type IL-2. The IL-2 variant stimulates immune cell proliferation and activation, which results in NK cell mediated tumor cell killing without the toxicity associated with binding CD25 and activating T regulatory cells. Clinical studies are anticipated to start during the first half of 2014.

Following the lunch break, Darko Skegro (Glenmark Pharmaceuticals SA) discussed the BEAT Platform, which is a “Plug & Play” approach to bispecific antibodies. The BEAT (Bispecific Engagement by Antibodies based on the T cell receptor) technology platform uses engineering of CH3 interfaces to mimic the natural association of T cell surface receptors α and β. By mutating the interface of the CH3 domain pairs, new heterodimeric interfaces can be built that facilitate heterodimer formation. The overall structure and glycosylation pattern is identical to that of an IgG1 Fc. Forming heterodimers of the Fc region enables the direct conversion of any antibody pair to a bispecific antibody with normal effector function and PK. In silico immunogenicity prediction algorithms demonstrate that the overall potential immunogenic risk is low for these novel heterodimeric bispecific antibodies. Characterization of a bispecific HER2/CD3 antibody using this technology demonstrated potent in vitro tumor cell killing mediated by effector T cells. Anti-tumor activity was also demonstrated in a breast cancer tumor model where human peripheral blood mononuclear cells (PBMCs) were mixed with the trastuzumab-resistant JIMT-1 breast tumor cell line. In terms of manufacturing, BEAT antibody clones expressed at up to 3 g/L with greater than 90% heterodimerization at harvest. The BEAT antibodies also demonstrated good stability over time and 97% heterodimers after protein A-based purification. The use of BEAT technology enables the production of heterodimeric bispecific antibodies that retain Fc effector function and the biophysical properties of natural IgGs.

Ulrich Brinkmann (Roche) gave a talk on multispecific antibody formats for cancer treatment that described the various formats of bispecific antibodies, as well as the challenges of retaining function and potency of multiple antigen binding components. The production of bispecific antibodies that are activated by proteolytic processing was highlighted, and Dr Brinkmann discussed examples of trivalent antibodies where the linker between the CH3 domains and the VL or VH domains contains a protease cleavage site. The cleavage of one chain of the linker essentially opens up the binding domain in a protease-specific manner that enables the activation of binding component of the multispecific antibody in a tissue-restricted fashion. He also discussed bivalent antibodies that use the knobs-into-holes method combined with domain exchanges to form bivalent “Crossmabs” that can bind two unique antigens. An example of an Ang2-VEGF targeting Crossmab that is currently in Phase 1 clinical study was used to illustrate the functionality of both targeting domains. Lastly, the use of a hapten binding bispecific antibody was described as a way to deliver targeted payloads in a modular approach. The hapten binding portion of the bispecific antibody can bind and deliver multiple payloads. The payloads include cytotoxic molecules, as well as siRNA, peptides, nanoparticles and fluorophores. Dr Brinkmann provided examples of hapten-containing nanoparticles that enclosed CD31 siRNA targeted toward VEGFR2 expressing cells of tumor vasculature. The various formats of multispecific antibodies described during this talk may enable a more targeted approach to modulate pathways in a tissue-specific fashion and the use of hapten-binding bispecific antibodies for targeted delivery of a variety of payloads.

Roman Kischel (Amgen) discussed the developing pipeline of therapeutics utilizing bispecific T-cell engager (BiTE) technology and provided an update on blinatumomab and other BiTE antibodies. The final speaker of the session, Eugene Zhukovsky (Affimed), then described bispecific TandAbs® that recruit NK and T cells to treat cancer. The TandAb® platform generates tetravalent bispecific antibodies that use any Fvs, independent of their origin. These unique bispecific antibodies are expressed from a single gene whose protein product homodimerizes and is bivalent for each target antigen. As an example, Dr Zhukovsky discussed AFM13, a TandAb that recruits effector cells through a
CD16A binding arm, and targets tumors through CD30. A unique feature of the anti-CD16 arm is its binding specificity for CD16A and not CD16B. This anti-CD16 arm displays subnanomolar binding to both CD16A allotypes (V/F158), and higher effector cell cytotoxicity compared with traditional IgG antibodies. In addition, the TandAb does not activate effector NK cells in the absence of tumor antigen engagement. AFM13 has completed Phase 1 clinical studies, with clinical activity demonstrated at 1.5 mg/kg and higher doses in Hodgkin lymphoma patients. AFM13 is equally active in patients who are refractory to brentuximab vedotin and those that have never received this recently approved anti-CD30 ADC. Another example of a CD19 and CD3 targeting TandAb (AFM11) was also presented. Potent preclinical activity was demonstrated with activation of both CD4 positive and CD8+ T cells, and no T cell activation or cytokine release was observed in the absence of binding to target cells. Activity of AFM11 was demonstrated in a Burkitt lymphoma xenograft PBMC admixed model with half-maximal inhibition of tumor growth observed at doses as low as 5 ug/kg. Complete suppression of tumor growth was seen at doses of 5 mg/kg. Clinical studies for AFM11 are planned for 2014. The use of TandAbs to treat cancer may enable the potent activation of effector cells in a tumor-specific fashion with a therapeutic that engages both the tumor and effector cells in a bivalent fashion.

Track B: Naked Mabs, Antibody Chemical Manufacturing And Controls

Randall J Brezski

Chaired by Randall Brezski (Janssen R&D), the Track B session on Wednesday focused on generating therapeutic naked antibodies, antibody analytics and chemical manufacturing and controls for antibodies. Inge Verbrugge (The Netherlands Cancer Institute) discussed the efficacy and underlying mechanisms of radiotherapy combined with immunotherapy for the treatment of cancer. Dr Verbrugge opened her talk with a review of how established tumors generate immunosuppressive factors that facilitate evasion of host immune responses by suppressing immune effector cells such as macrophages, NK cells, and T cells. She then presented a brief overview of immunomodulatory molecules available at the Netherlands Cancer Institute, including T cell receptor gene transfer, tumor infiltrating lymphocyte (TIL) therapy, and antibody-based immune modulation, with the remainder of the talk focusing on the latter category. Dr Verbrugge provided two examples of antibody-based immune-modulation. The first involved blocking T cell co-inhibition pathways through the use of antibodies such as those directed against PD-1 or CTLA-4, which are thought to “release the break” on immune responses. The second category involved administering antibodies to facilitate T cell co-stimulation (e.g., anti-CD137), which could “press the gas” and drive immune responses. However, Dr Verbrugge indicated that while single agent immunotherapy is promising, it may be suboptimal. She then discussed combining immunotherapy with localized (external beam) radiotherapy as a potential solution.

Dr Verbrugge presented data from an AT-3 mammary tumor model and showed that the tumor cells expressed PD-L1, the ligand for PD-1, whereas PD-1 was expressed on a large proportion of TILs. Within the TIL population expressing high levels of PD-1, a subset of those expressed the costimulatory receptor CD137. To optimally allow (co-)stimulation of T cells, immunotherapy was designed using a combination of anti-PD-1 and anti-CD137 monoclonal antibodies and was used alone or in combination with single-dose or fractionated radiotherapy. While radiotherapy and immunotherapy as single agents delayed tumor outgrowth, only combined treatment was able to cure mice bearing established orthotopic AT-3 tumors. Using antibody-based depletion of CD4-positive T cells, NK cells, or CD8β-positive T cells, she subsequently demonstrated that CD8β-positive T cells were crucial for the curative effect of the combined therapy. In addition, tumor-specific CD137-expressing T cells were enriched in the tumors following radiotherapy, indicating that radiotherapy does not deplete, but rather enriches the tumor microenvironment of tumor-specific effector cells. A follow-on study indicated that inhibition of signaling through the mammalian target of rapamycin (mTOR) pathway using everolimus during the first 10 d of treatment almost completely abrogated the curative effect of the combination of radio- and immunotherapy. This was explained, at least in part, by reduced MHC class I expression on tumor cells, reduced dendritic cell activation status, and reduced CD8-positive T cell functionality.

William Finlay (Pfizer) presented a study on engineering scFv-based bifunctional molecules for subcutaneous delivery. He first reviewed the myriad of bispecific formats, and then discussed the merits of a fairly simple scFv-Fc-scFv format. The pros for this format include the need for only a single polypeptide, a single expression/purification path, no light chain swapping, and a single formulation; cons included stability issues associated with scFv constructs, unpredictable behavior at high concentrations, and the inability to control dosing of each partner. Additionally, bispecific molecules with inherent scFv instability could limit drug administration to slow intravenous infusion or intraperitoneal delivery, since subcutaneous dosing would require scFv-containing constructs amenable to high concentration formulations.

Dr Finlay then presented a case study on a bispecific scFv-Fc-scFv targeting Immunomodulatory Target 1 (IT1) and CXCL13. Initial screening of the IT1 x CXCL13 domain bispecific molecule revealed that, although the IT1 domain was stable and well-behaved, the scFv anti-CXCL13 was unstable and had fast off-rates for both human and cynomolgus CXCL13. Dr Finlay then described a V3’βV1’ βCDR3 mutagenesis approach coupled with a “hammer-hug” selection process that was designed to enrich for clones having off-rates in the low pM range coupled with a thermal-selection branch designed to enrich for more stable clones. The selection process was also performed in the presence of positively charged histones to reduce the enrichment of clones with excess negative-charge mutational bias, due to charge-based interactions as a result of the positively-charged nature of the CXCL13 antigen. Disregulated charge in the antibody binding
interface has been shown to be a risk factor for polyspecificity and poor PK.

The results yielded clones with improved potency, and it was noted that only four mutations in the CDR-L3 were required for the increased potency. Dr Finlay then discussed a biophysical assessment of optimized anti-CXCL13 variants using a thermal ELISA assay. This approach involved heating a fixed concentration of clones at 60 °C for 1 h, followed by centrifugation to remove aggregated material, then assessment of antigen binding by ELISA of the heat-stressed material compared with untreated controls. The thermal ELISA approach coupled with other assessments, such as cross-reactivity to related chemokines and forced aggregation assays, allowed a more refined selection of top-performing clones. Ultimately, the top-performing clones selected under these criteria demonstrated expression levels consistent with a standard IgG1 mAb, stability at 100 mg/ml concentrations, and viscosity similar to IgG1 mAb controls. Dr Finlay concluded that the Octet provided a high throughput assay system to quantify IgG levels from hybridoma supernatants, as well as monitoring IgG concentrations at different time points after transfections. Finally, the Octet was used to support NovImmune’s κλ bodyTM bispecific platform. A κλ bodyTM is a fully human IgG with no mutations or linkers which shares a common heavy chain and contains two different specificities driven by distinct light chains, one lambda and one kappa. Ms. Malinge noted that the Octet proved useful in identifying stable cell lines that express similar levels of kappa and lambda chains and thus maximize assembly of the bispecific molecule.

Following the lunch break, the focus of the afternoon Track 2 session was on antibody chemical manufacturing and controls (CMC). Hervé Broly (Merck Serono SA) discussed a high-throughput media design approach for high performance mammalian fed-batch cultures. Dr Broly indicated that the biopharmaceutical industry is entering a rapidly changing environment that includes increased regulatory expectations, the emergence of biosimilars, and the potential for financial crises. Due to these pressures, the industry is expected to develop new biopharmaceutical entities (NBE) faster, yet with decreased costs. To meet these demands, Dr Broly noted that Merck Serono applies a Technology Platform concept to reduce timelines from gene to material available for first-in-human use, to optimize large scale capacities designed for fed-batch-based manufacturing processes, and to lower costs by process standardization.

Dr Broly then discussed an upstream technology platform approach as a standardized manufacturing process amenable for mid- and large-scale capacities. This included a CHO-designed fed-batch approach where the cell culture volume is expanded gradually with a proprietary chemically-defined cell culture medium for ultimate delivery to the production bioreactor, where the cell are then cultured under predefined process parameters. Dr Broly concluded his presentation by discussing a 96-deep well plate high throughput technology for developing media and feeds. A case study was presented where multivariate analysis was used to identify key components that influenced the performance of cell lines. The 96-deep well plate technology was then used to assess the capability of six different cell lines to change relative to their environment by modulating six factors identified to have an effect on product quality. The results demonstrated that each cell line reacted differently to environmental changes. Dr Broly indicated that this methodology allows the rapid development of high performance media and feeds.

Ralf Holzinger (CONFARMA France SARL) presented test models for advances and developments in safety for biologicals. He indicated that there are many risk factors that can contribute to clinical trial failures up to Phase 3 due to serious adverse events, including infusion reactions, anaphylactic shock, fever, pain, immune and allergic reactions, and cytokine storms. For instance, the induction of cytokine storm in the clinic by the anti-CD28 mAb TGN1412 is indicative of the serious consequences and potential unexpected responses to the use of biotherapeutics in humans. Therefore, advances in safety assays during the development of biotherapeutics can potentially accelerate the preclinical process and mitigate safety risks. Mr Holzinger then reviewed how the presence of harmful pyrogens in biotherapeutics can trigger toll-like receptors (TLRs) on immune cells such as monocytes and macrophages, resulting in the induction of cytokines. Monocyte Activation Test (MAT) systems are often employed to detect the presence of pyrogens, and these assays can be performed with whole blood, PBMCs, or monocyte cell lines. Mr. Holzinger then discussed the Mono Mac 6 cell line, which was established from PBMCs of a donor with unlimited secretion of IL-6. Mr. Holzinger indicated that the use of the Mono Mac 6 cell line for the detection of pyrogens is suitable for the analysis of biologic products, including blood products, vaccines, recombinant proteins, and antibodies.

Mr. Holzinger then discussed other safety factors, including the presence of host cell impurities such as DNA and proteins. Residual host cell protein and DNA quantities contained within the final dosage form must follow regulatory guidelines established by the World Health Organization, FDA, and European Medicines Agency. He discussed a universal assay for the detection of any eukaryotic DNA using a qPCR reaction
universal for all host cell DNA, so that one single assay could be used for all cell lines, from yeast to human, including all mammalian cell lines often used for the expression of mAbs. Mr. Holzinger indicated that as the pharmaceutical industry trends toward increasing numbers of biological molecules, the incorporation of these types of safety assays can comply with regulatory guidelines and potentially alleviate risks associated with the use of biologics in humans, thereby increasing patient safety and accelerating development of biologics and mAbs.

The last presentation of the session was from Richard Kensinger (Sanofi Pasteur), who discussed PEGylation optimization of a human engineered Fab’ fragment. PEGylation is frequently employed to increase the hydrodynamic volumes of small proteins, which can in turn favorably impact PK properties by increasing half-life. At present, there are eight licensed pegylated proteins, including the anti-TNF Fab certolizumab pegol. Dr Kensinger indicated that a key consideration in the pegylation reaction described was controlling the amount of PEG-maleimide added in ratio with the hinge region sulfhydryl groups of the Fab. The desired di-pegylated product requires an excess of PEG-maleimide to be added to the reaction in order to minimize the presence of mono-pegylated product, which may have less favorable PK properties compared with the di-pegylated product. To optimize the pegylation reaction, Dr Kensinger noted that a Quality by Design (QbD) approach was used to optimize di-pegylation. They found that an excess amount of PEG-maleimide maximized the formation of the di-pegylated product and minimized the amount of mono-pegylated by-product. However, the high cost of PEG-maleimide necessitated optimization of the pegylation reaction prior to scale-up. Utilizing a factor-prioritization risk assessment tool, Dr Kensinger indicated that the most important factors in the pegylation reaction were narrowed down to the critical few to be studied by the design of experiment (DOE) process. Reaction optima were identified and verified around the optimal reaction ranges, followed by a comparison of the DOE optimized conditions with the starting manufacturing process conditions. Dr Kensinger showed that increased Fab concentrations and decreased amounts of PEG-maleimide added yielded ~25% PEG recovery, while at the same time maximizing the di-pegylated product. The verification of optimal reaction conditions can then be applied to investigational new drug application filings and aid the manufacturing process.

Looking Forward: The Reality of mAb Biosimilars

Alain Beck

The closing plenary session of the 9th EAC was shared with the co-located 2nd World Biosimilar Congress. Chaired by Alain Beck (Pierre Fabre), the session consisted of two presentations discussing the characterization and the reality of mAb biosimilars. In the first presentation, Sarah Fredriksson (Genovis) provided an overview of several unique enzymes isolated from Streptococcus pyogenes that cleave IgGs at selected sites. The usefulness of Genovis’ enzymatic toolbox was demonstrated for antibody, Fc-fusion proteins and ADCs fragmentation and N-deglycosylation characterization by MS. The immunoglobulin-degrading enzyme of S. pyogenes (IdeS), also called FabRICATOR, is a genetically modified enzyme. IdeS cleaves IgGs under their hinge domain in a GG amino acid motif, yielding F(ab′)2 and Fc antibody fragments. After reduction, three 25 kDa fragments are obtained (light chain, Fd and half Fc). In contrast to other enzymes, IdeS cleaves many IgGs (IgG1, 2, 3 and 4; mlg2a and 3; rlgG) at exactly the same site in a very short time, and each antibody is cleaved only once. The approach has the advantage of being rapid (less than 2 h for the entire analysis including digestion and LC-MS analysis), informative, and low material consuming. IdeS helps accurate profiling of N-glycans site by site. This is particularly interesting, for example, in the case of the presence of Fab glycosylation such as for cetuximab because the two glycosylation sites will be separated on two different fragments. As a consequence, the extent of fucosylation and galactosylation on the Fc, known to directly act on effector functions, can be monitored more efficiently. In addition, various charge and size variants such as C-terminal lysine cleavage, N-terminal pyroglutamination, oxidation, truncation, and incompletely processed signal peptides can be resolved and characterized.

The use of IdeS is becoming increasingly popular for the fast characterization of antibody by MS, including correct sequence assessment, antibody Fab and Fc glyco-profiling, biosimilar comparability studies and Fc-fusion protein studies, as well as ADCs. LC-MS analysis of ADCs is becoming an increasingly important tool to characterize the changes in DARs of ADCs in patients. To quantify the proteolytic degradation of ADCs via LC-MS, fragmentation of large molecules into smaller pieces is required. In summary, IdeS proteolytic digestion is rapidly becoming a reference analytical method at all stages of antibody and antibody-related product discovery, preclinical and clinical development. The method is routinely used for comparability assays, formulation, process scale-up and transfer, and to define critical quality attributes (CQA) in a QbD approach.

Dr Fredriksson also discussed several other useful enzymes, including EndoS endoglycosidase (IgGZERO), which specifically and rapidly cleaves N-linked glycans from antibodies, leaving one N-acetyl-D-glucosamine and one fucose. Removing the sugar molecules can improve the performance of the antibody in various applications. Glycan can be analyzed separately and used for characterization of antibodies. IgGZERO can also help wash away antibody molecules from primary cells. IgGZERO does not cleave high mannos structures and bisected glycans. Interestingly, this may be achieved by another enzyme (EndoS2, GlycINATOR) following a simple protocol and mild reaction conditions. Cysteine protease SpeB (FabULOUS) allows upper hinge digestion of antibodies. Based on sequential digestion of an IgG1 by SpeB and IdeS, Dr Fredriksson and her colleagues were able to detect the released hinge peptide; MS/MS data suggested that the SpeB cleavage site is situated upstream from the IdeS cleavage site.

Gabriele Reichmann (Paul-Ehrlich-Institut; European Medicines Agency) discussed biosimilar mAbs in the European Union, and included updates on EMA guidelines, future
directions and development. For the EMA, a biosimilar is a version of the active substance of an already authorised original biological medicinal product.\textsuperscript{47} Demonstration of similarity in terms of quality characteristics, biological activity, safety and efficacy based on a comprehensive comparability exercise must be provided.\textsuperscript{44} The aim is to generate a molecule as similar to the reference product as possible to rely on the safety and efficacy proven for the reference product. The easiest approach is to use the same pharmaceutical form and strength, and the same route of administration as for reference product. Deviations of formulation or excipients are justifiable, but require justification or further studies. Intended changes to improve efficacy (i.e., biobetter) is not compatible with the biosimilarity paradigm. A basic requirement is to use a reference medicinal product authorized in the European Economic Area (EEA) on the basis of a complete dossier. A single reference product should be used as the comparator throughout the comparability program; a non-EEA reference product may be accepted to facilitate global development and to avoid unnecessary clinical trials. A practical way is to use batches sourced from outside EEA in certain studies (e.g., clinical efficacy study) if the non-EEA comparator is licensed in region with similar scientific and regulatory standard (i.e., ICH) and if the applicant has established that the non-EEA product is representative of the product authorized in EEA. In this case, the bridging of data are needed, including

in vitro analytical results (structural, functional) and possibly clinical PK or PD data. Acceptability of non-EEA product should be discussed as early as possible with regulatory authorities. The biosimilar concept is applicable in principle to any biological medicinal product. In practice, however, biotechnology-derived proteins and immunological products (e.g., vaccines, allergens) are likely candidates. More complex products (e.g., gene or cell therapies) are unlikely candidates, but may be considered in the future on a case-by-case basis, in light of scientific knowledge and regulatory experience gained over time.

In 2008, it was not obvious to imagine biosimilar mAbs because of perceived quality issues that might result from the complexity of the molecules.\textsuperscript{49,50} The situation is completely different in 2013, with numerous biosimilar mAb and fusion proteins\textsuperscript{51} in clinical trials in the European Union. The marketing authorization in the EU for the first biosimilar mAb, Remsima / Inflectra (infliximab), definitively demonstrate that it is possible to bring biosimilar mAbs on the market in highly regulated countries.\textsuperscript{52} Dr Reichman discussed a number of case studies, such as the shift in the quality profile of reference product\textsuperscript{8,10,53} and how to define the biosimilarity margin. For example, changing of the expression system for biosimilar (e.g., CHO to Pichia pastoris)\textsuperscript{34} results in significant differences in glycosylation profile.\textsuperscript{55,56}

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