Drugs of the Future

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Clarivate Analytics

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Meeting Report

18th Annual PepTalk: The Protein Science Week – Antibody–Drug Conjugates: Next-Gen Engineering

San Diego, California, USA – January 15-16, 2019

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Summary

The Cambridge Healthtech Institute's (CHI) 18th Annual PepTalk Conference was dedicated to proteins, covering their engineering, production and applications in medicine. It comprised seven parallel tracks: 'Protein Engineering & Development', 'Antibody Therapeutics', 'Innovations in Discovery and Development', 'Formulation & Stability', 'Analytics & Impurities', 'Process Technologies & Purification', and a mixed stream addressing 'Biotherapeutic Expression & Production' and 'Alternative Expression & Products'. Approximately 1,300 participants attended the various sessions. The event also included so-called 'Buzz Sessions', in which attendees gathered in roundtable discussions to address highly specialized topics. Additionally, more than 150 posters were on display, located close to an exhibition with approximately 100 booths of various suppliers for protein research. This report covers the 6th Antibody–Drug Conjugates conference with the subtitle 'Next-Gen Engineering', which was held as part of the 'Antibody Therapeutics' stream.

Key words: IMGN-779 – IMGN-632 – XMT-1522 – XMT-1536 – HDP-101 – Rovalpituzumab tesirine – Cofetuzumab pelidotin – Trastuzumab – Rituximab – Tesirine (SG-3249)

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Fighting Cancer with ADCs

In his Keynote Presentation, Rakesh Dixit (MedImmune) discussed the advances of next-generation antibody–drug conjugates (ADCs), immunotherapies and combinations in the war against cancer. Besides a new wave of ADCs, other modalities, such as cancer vaccines, enhanced antibody-dependent cellular cytotoxicity (ADCC), immunotoxins, T-cell engagers, oncolytic viruses, checkpoint inhibitors and the classical treatments (chemotherapy and radiation) should not be forgotten. Currently, four ADCs are approved: **brentuximab vedotin** (Adcetris), **gemtuzumab** (Mylotarg), **trastuzumab emtansine** (Kadcyla) and **inotuzumab ozogamicin** (Besponsa). In the case of immunotoxins, **moxetumomab pasudotox** (Lumoxiti), against hairy cell leukemia, was launched in 2018 in the U.S. Currently, more than 55 clinical trials with a wide range of modalities are ongoing, 9% of which are in phase III. Despite the impressive features of ADCs, such as their superiority in rapid tumor regression, durable responses and long overall survival, they still suffer from complicated manufacturing and the difficulty to differentiate between efficacy and safety. Repeated dosing at lower concentrations reduces toxicity. The current ADCs already represent the third generation of molecules as they combine many improvements (e.g., site-directed conjugation, improved linkers and drugs). Additionally, new modes of action are being established, such as biparatopic antibodies binding their target at two different epitopes, thus enforcing crosslinking and efficient internalization. Other approaches allow binding at lower pH in the tumor environment, differentiating them from target presence on normal cells. Translational strategies focus on maximizing the therapeutic index and careful selection of patients by biomarkers. Some newer conjugates (pyrrolobenzodiazepine [PBD]-ADCs) even trigger a memory response, involving cytotoxic T-lymphocyte (CTL) contribution. In a look at the reasons for discontinuation, 24% of the programs with ADCs were closed for safety and the same percentage for efficacy reasons. Unsolved issues with these treatments in

translational science are large tumors, heterogeneous antigens, sick patients with suppressed immune systems and off-target effects due to binding to normal tissues.

ADCs with an IGN (indolinobenzodiazepine pseudodimer) payload in hematologic malignancies were the topic of the presentation by Yelena Kovtun (ImmunoGen). Acute myeloid leukemia (AML) is the deadliest blood cancer with less than 30% survival after 5 years and often relapse with multidrug resistance. The new drug mono-imine containing IGN exerts two functions: alkylation and crosslinking. The ADC **IMGN-779** containing the IGN derivative DGN-462 is conjugated via a disulfide linker to the monoclonal antibody (MAb) Z4681A and targets CD33 with picomolar affinity. IMGN-779 exhibited strong antitumor efficacy in various AML xenograft disease models, and achieved durable tumor regressions and prolonged survival. **IMGN-632** is directed against CD123 on B-cell acute lymphoblastic leukemia/lymphoma (B-ALL) cell lines and primary B-ALL blasts. IMGN-632 containing the humanized MAb G4723A efficiently eliminated more than 90% of lymphoma cells at IC_{50} values below 20 pM. Interestingly, IMGN-632 displayed potent activity against AML cells without affecting normal bone marrow progenitors, indicating a potential to cure AML patients in the absence of or with limited myelosuppression.

Jared Spidel (Eisai) explained the simple and efficient production of homogeneous, site-specific ADCs with transglutaminase. Initially, potential conjugation sites in the form of lysines were screened according to the hypothesis that native IgG lysines could act as acyl acceptor sites for glutamine-based acyl donor substrates. In human IgG more than 80 lysines are present of which 40 to 50 are found in solvent-exposed loops. An ELISA-based assay was developed to identify positions amenable for transglutamination. Interestingly, none of these lysines were accepted as substrate, and thus it was investigated if the C-terminal lysine at position 447 could serve as target. Unfortunately, this lysine is cleaved by the activity of carboxypeptidase B in cell hosts. Alternatively, a known acceptor peptide with the sequence LSLSPGKGGSTKHKIPGGS was fused to the C-terminus of the heavy chain of an IgG, which resulted in transamidation of all three lysines by transglutaminase. The newly introduced lysine at position 447 is the ideal target for enzymatic conjugation if followed by any nonacidic, non-proline amino acid residue at position 448. The additional lysine can now be conjugated either in a direct or sequential manner to add linkers and payload at the C-terminus of the heavy chain resulting in a perfect drug:antibody ratio (DAR) of 2. Light chains can be modified utilizing the additional disulfide bond existing in rabbit light chains between positions 80 and 171. Introducing only the point mutations at C80 delivers a site-specific conjugation position for thiols in the light chain. Both techniques with an extra lysine at the heavy chain and an extra cysteine in the light chain allow for exact DARs of 4.

Jan E. Schnitzer (PRISM) described how transvascular pumping of ADC into solid tumors boosts drug potency and safety. A typical limitation of current ADC therapy is the need for too high dosing, as most of the drug is distributed elsewhere. Particularly in solid tumors, the toxicity limits the efficacy because only passive transvascular transport delivers the ADC to the tumor. Slow diffusion over the extracellular matrix reaches only tumor endothelial cells exposed to the blood. In order to improve tumor-targeted transport a search for molecules with high expression in the caveolae was started. Crosslinking membrane-bound proteins in the caveolae to cationic colloidal silica allowed for their subsequent identification by proteomic techniques. Doing this, a post-translationally modified form of annexin A1 (AnnA1) was found, which was selectively concentrated in caveolae. Raising an antibody against that target allowed for the efficient translocation of the antibody across the endothelium into a variety of tumors. This approach increased the antibody levels in the tumor by a factor of more than 100. The antibody was actively pumped into the tumor, which constitutes a new therapeutic approach for drug delivery in the form of ADC into a solid tumor.

Dimiter Dimitrov from the University of Pittsburgh discussed ADCs targeting tumor stromal cells, which seems to be a promising approach utilizing antibodies against the transmembrane receptor TEM8 (tumor endothelial marker 8). TEM8 is present on cancer-associated fibroblasts, endothelium and pericytes at high expression levels. Antibodies against that target conjugated to monomethylauristatin E (MMAE) eradicate malignant cells by a novel mechanism called "drug activation and release through stroma", which concentrates the drug by the tumor microenvironment. The drug is released without internalization, which results in a massive bystander effect that eliminates cells in proximity. Another promising target expressed on multiple cancer cell types and tumor-infiltrating blood vessels is CD276/B7-H3. An antibody with the payload PBD successfully kills both cell types leading to removal of large metastases and improved long-term survival.

Next-gen Engineering Strategies for ADCs

In a featured presentation, Marc Damelin (Mersana Therapeutics) gave considerations and examples of next-generation ADCs. The key elements of a successful ADC consist of a specific target, a selective antibody, a functional linker and a toxic payload. The currently addressed targets represent a broad range of expression profiles. Ideally, targets are chosen taking into account the dependencies of biophysical properties, biological functions and clinical development strategies. This also includes translational approaches looking at animal models and homologies between species. Biodegradable polymer molecules in the form of 'Fleximers' allow for DARs of more than 20. High hydrophilicity can accept even highly hydrophobic

payloads. The platform technology Dolaflexin increases efficacy, safety and tolerability, which is achieved by a specific linker that conjugates the Fleximer, loaded with multiple drug molecules with the antibody at multiple sites. The good solubility of that new macromolecule prevents aggregation, one of the main complications during manufacturing. For instance, the ADC **XMT-1522** (targeting a HER2 epitope different from trastuzumab) contains auristatin conjugated at a DAR12 with the conjugation technique Dolalock. Here the cell permeable payload is partially released by proteases in the tumor microenvironment while the uptake into cancer cells is mediated by the internalization of the ADC. Intracellularly the drug is converted into a non-cell permeable molecule killing only cancer cells. A combination of bystander effects leads to a highly specific killing of cancer cells and neighboring cells. The ADC **XMT-1536** targeting the surface molecule sodium-dependent phosphate transport protein 2B (NaPi2b) is currently in a phase I clinical study.

Stefan R. Schmidt (BioAtrium) discussed the benefits and limitations of different therapeutic modalities by comparing conjugated and engineered antibodies. Despite the successes of ADCs, with four molecules now approved, there are still a number of issues to be solved. A general problem is the unspecific toxicity that affects hepatocytes and causes neutropenia and other negative effects on hematopoietic cells. Interestingly, most of these reactions are caused by labile or cleavable linkers and can be eliminated relatively easily. Better efficiencies of ADCs can be achieved by utilizing bispecific antibodies that either improve internalization through crosslinking of biparatopic binding or increase the level of lysosomal targeting by simultaneously binding a cancer antigen and a lysosomal marker. Further optimization can be obtained by miniaturizing the binding domain, using antibody fragments or small scaffolds that penetrate the tumor deeper. Covering the antibody binding sites by peptide caps reduces unspecific toxicity as the cap is only released by proteases in the tumor environment. Tumor killing by antibodies can also be achieved without any additional payload but by protein engineering increasing the binding to FcγR to engage ADCC activity. Another cancer toxicity via an internalization pathway is possible using fusion proteins with toxins, kinases, proteases or RNases as fusion partners. Approaches that benefit from the engagement of immune cells utilize bispecific antibodies or immunocytokines. A clear advantage of fusion proteins is the simple manufacturing process that does not require any interruption for a chemical conjugation.

George Badescu (Heidelberg Pharma) presented amanitin-based ADCs as new therapeutic modalities for cancer therapy. Amanitin, a bicyclic octapeptide from mushrooms, is a highly potent cytotoxin that inhibits RNA polymerase II. In the unconjugated form it is highly toxic to liver cells as it is actively transported via solute carrier organic anion transporter family member 1B3 (OATP1B3) into hepatocytes; due to this, amanitin is very effective against low copy number targets (for instance, B-cell maturation antigen [BCMA] with less than 3000 copies per cell). Currently, a new synthetic pathway has been designed that creates the active molecule in 36 reaction steps. This also works under GMP (good manufacturing practice) conditions and allows modifications to incorporate further optimization and fine-tuning of its toxicity. The toxin kills quiescent and dividing cells alike, and does not generate a bystander effect as it is not taken up in normal cells due to its low permeability. **HDP-101** is the first ATAC (antibody-targeted amanitin conjugate) directed against BCMA and expected to enter phase I trials for multiple myeloma by the end of 2019.

Honing in on ADC Targets

The targeting of tumor-initiating cell (TiC)-associated antigens with ADCs was described by Alex Bankovich from AbbVie Stemcentrx. TiCs are the starting point of most tumors and represent an important cell type to eliminate in order to prevent relapses. Early targets affecting cell fate decisions are delta-like protein 3 (DLL3; Notch pathway), tyrosine-protein kinase 7 (PTK7; Wnt signaling) and Ephrin-A4 (EFNA4; Ephrin pathway). DLL3 usually retained inside cells is found on the surface of small cell lung cancer (SCLC) TiCs. The ADC **rovalpituzumab tesirine** (SC16LD6.5, Rova-T), with a DAR of 2 utilizing a PBD dimer and a cleavable linker, depleted TiCs in SCLC patient-derived xenografts. Combining rovalpituzumab tesirine with checkpoint inhibitors improved the sustained effect. Currently, rovalpituzumab tesirine is in several clinical studies (phase I to III) in several SCLC patient groups. PTK7 is overexpressed in cancer stem cells both in tumor and stroma. Using auristatin as an ADC warhead in non-small cell lung cancer or ovarian carcinoma tumor models showed good responses. As PTK7 can also be found on dendritic cells, it was speculated that immune suppressive effects can be eliminated by killing PTK7-carrying dendritic cells. **Cofetuzumab pelidotin** (Pfizer/Stemcentrx), an ADC comprising the anti-PTK7 antibody PF-06523435 linked to the cytotoxic auristatin PF-06380101, is in phase I studies.

Jennifer Hill (National Research Council Canada) explained the utilization of multi-omics data and functional screens to select ADC targets. A big emphasis on ADC development must be put on target selection and candidate drug design; in particular, target selection can be supported by omics-based data mining. The minimal requirements for a good target are high expression in diseased versus normal tissue, cell surface localization and internalization. Microarray and RNA sequencing data from more than 45,000 different human samples were assessed and quantified. Due to the heterogeneity of tumors, patient stratification is required to improve target selection, and including exon analysis to identify aberrant splicing helps in increasing specificity. Surface labeling and subsequent proteomics analysis

identifies cell surface-located targets. Proteomics and labeling is also the method of choice to resolve the cellular trafficking of the targets. The internalization is verified in a cell-based screening that evaluates more than 600 different conditions; a hit is considered positive if the IC_{50} is below 1 nM. Currently, 49 different targets with at least 2-fold overexpression are addressed in various preclinical developments.

Julian Andreev (Regeneron Pharmaceuticals) revealed how to select potential ADC targets by a proteomics screen. One limitation of ADC is the requirement of internalizing targets to get the toxic payload into the malignant cell; alternatively, constitutive lysosomal internalization can be exploited to get active warheads into the cytosol. So far, prolactin receptor (PRLR) has been the protein of choice to use in a bispecific ADC approach: one arm binds the cancer target, while the other IgG arm connects to PRLR that drives lysosomal targeting and ultimately the release of the payload. This mechanism also allows for cancer surface antigens to be addressed that would normally not be internalized, thus greatly expanding the possibilities of target selection. Another high-turnover protein, amyloid-like protein 2 (APLP2), can be used according to the same principle. This new approach, combining two unrelated targets, significantly improves the efficacy of ADCs, even those targeting internalizing antigens such as HER2.

A new technology for site-specific conjugation for next-generation ADCs was described by Tatsuya Okuzumi (Ajinomoto). The technology is based on a highly specific 13-amino acid peptide that binds to the hinge region of the Fc-domain of human IgG at an affinity of 20 nM. Further improvement and the addition of 4 amino acids improved the affinity to 4 nM. The peptide with the conjugated linker is called AJICAP reagent and can be directly utilized for site-specific conjugation to the IgG at lysine 248. The resulting ADC displays an ideal DAR of 2. AJICAP is compatible with IgG1, 2 and 4. Variants of **trastuzumab** and **rituximab** have been used as material for proof-of-concept studies with a wide range of different payloads. Conjugated antibodies still show a normal FcRn binding that supports long half-life. Dosing of 5 mg/kg inhibited tumors in a similar way to trastuzumab emtansine but with an at least 2-fold higher maximum tolerated dose.

Improving Site-specific Conjugation, Payloads and Scaffold Engineering

Yuan Cheng (Amgen) described the development of a robust site-specific conjugation platform for hybrid molecules. In the design of ADCs, site-specific conjugation and the chemistry of the linkage play an important role. Typically, lysines or cysteines have been used successfully. In the presented study cysteine point mutations were distributed over the whole protein molecule. Altogether, 267 residues were selected, but positions and amino acids important for structure and function (e.g., glycine, proline, complementarity determining regions and CH3 domains) were excluded from the mutagenesis. In 183 cases the substitution was successful; however, the major problem was antibody dimerization. Cystamine capping could be introduced to prevent multimerization. Finally, 13 mutants were screened for their conjugation efficiency. Up to 500 samples could be conjugated simultaneously while applying mass spectrometry with a processing time of less than 20 s to analyze the results. All these combined efforts led to the identification of three ideal sites in the IgG molecule that became part of the routine platform screening for new ADC molecules. So far, even the scale up to multigram conjugation has been successful.

Highly homogeneous ADCs based on dual variable domains (DVDs) were shown by Christoph Rader (Scripps Research Institute). The catalytic antibody 38C2 contains a deeply buried lysine in the binding pocket that is part of the catalytic functionality. The hapten used to select that antibody can be used as a starting point for covalent site-specific conjugation that takes place rapidly and efficiently at neutral pH in a single step. Grafting a second variable domain targeting HER2 to obtain the DVD molecule incorporates two functionalities: the new antigen binding and the specific conjugation site. The ADC variant containing monomethyl auristatin F showed high activity against xenograft models. Replacing the reactive lysine at position 99 by arginine generates the opportunity to implement a second, different payload in a single molecule. The final resulting molecule is then an orthogonal DVD-ADC with a constant light chain and heterodimeric pairing of the heavy chains by knobs-into-holes technology.

Philip Howard (MedImmune/Spirogen) gave an overview on antibody PBD conjugates. PBD dimers are currently highly popular in the context of ADCs; two variants, tesirine and talirine, have been most frequently used in clinical trials. **Tesirine** (SG-3249) was designed to combine potent antitumor activity with desirable physicochemical properties such as favorable hydrophobicity and improved conjugation characteristics. In general, PBD dimers cross-link DNA in a sequence selective fashion and block replication. Interestingly, the modified DNA is not addressed by nucleotide excision repair, creating a long-lasting effect. The drug can be attached to the linker at two different sites increasing the combinatorial possibility and compatibility with different conjugation techniques. Even the distance of the PBD monomers can be adjusted by reducing the number of C-atoms in the tether from 5 to 3, thus minimizing the binding into the DNA minor groove. Comparisons with other standard payloads, auristatin and maytansinoid, revealed a much higher potency at identical dosing of PBD dimers together with a longer-lasting effect.

Shalom Goldberg (Johnson & Johnson) described the design, characterization and LC-MS/MS bioanalysis of

protein–drug conjugates. The heterogeneous nature of ADCs complicates their in vivo analysis. Typically, the technologies employed include ligand-binding assays to prove the functionality of the antibody while monitoring the payload quantity by mass spectroscopic methods. To simplify assay development, a smaller surrogate of an IgG, the Centyrin, was used. The conjugated Centyrin was trimmed down to a peptide fragment by tryptic digest. In order to assess the hydrolyzation of the conjugate it was important to include succinimide hydrolysis products of the linker in the quantitation method as well. The most accurate measurement required the coupling of LC-MS/MS techniques to reveal all products and to ensure correct quantification, also from in vivo samples.

Disclosures

The author is an employee of Clarivate Analytics.