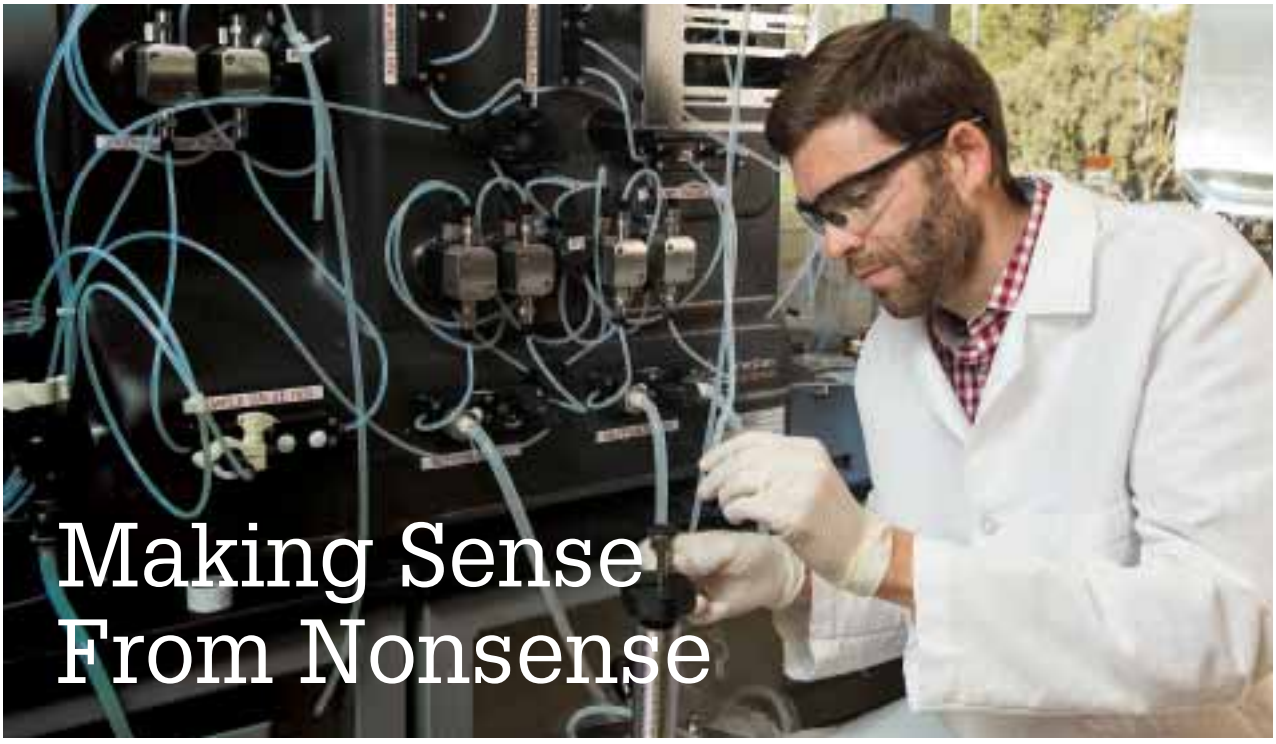

Making Sense From Nonsense

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New generation genetically engineered ADC technology can not only expand the genetic code, but is also capable of creating mammalian cell lines that produce protein products containing unnatural amino acids. The benefits for science are huge, including more design freedom when it comes to developing drugs with greater therapeutic potential

Feng Tian at Ambrx Inc

Antibody-drug conjugates (ADCs) can be regarded as the latest version of the 'magic bullet' cytotoxic drugs used in chemotherapy. 'Magic bullet' and 'chemotherapy' were terms first coined by Paul Ehrlich a century ago, when he was attempting to find small molecules that could target pathogen-specific receptors (1). While neither term was initially proposed for oncology, the idea of targeted therapy became rapidly popularised in cancer research due to its simple conceptualisation. After overcoming technical challenges, the first ADC was tested in cancer treatment 50 years later (2). Although unsuccessful, a new therapeutic paradigm was introduced, and ADCs have gained significantly more attention ever since.

In contrast to the original idea that the magic bullet is a small molecule, the modern magic bullet – an ADC – is a macromolecular combination of a large molecule (an antibody) and a small molecule (a drug) bridged by a linker. The antibody serves as the true magic bullet because it binds to specific antigens that are expressed on the surface of diseased cells. After being internalised into cells, ADCs release the drug to start the intracellular chemotherapy that allows targeted cells to be killed. It is therefore very important to keep the linker stable in the bloodstream and unstable in the cancer cell. In the time since this concept was introduced, scientists have been attempting to improve all three components to meet the unique capability of target-directed cancer therapy.

Evolution of ADCs

For production of first-generation ADCs, drugs are conjugated to the antibody's chemically active amino acid residues, such as lysine or cysteine. Although this is a convenient method for forming conjugations with standard existing antibodies, the end products are mixtures, because randomly reacting lysines are used each time during conjugation. It is estimated that 86 lysines reside in a typical immunoglobulin G antibody. When the drug-to-antibody ratio (DAR) is six, at least 40 out of those 86 lysines are modified during a typical N-hydroxysuccinimide ester conjugation (3). This results in a mixed product that potentially has 4.5 million different ADCs containing different lysines.

In the case of cysteine, only eight of the cysteines that are responsible for forming interchain disulfide bonds are used for conjugation. Furthermore, the conjugates have much less heterogeneity. Most cysteine conjugates often have DARs varying from 1.3 to 3.9 (4). It has been shown that the optimal DAR is 2~4, which translates into superior drug *in vivo* efficacy. However, it was recently revealed

that by reducing the hydrophobicity of the payloads or linkers, the optimal drug load can be shifted to a DAR of 8 (5-7). In the last seven years, second-generation (site-specific) ADCs have been developed to overcome the limitations of the first generation in heterogeneity, therapeutic index, toxicity and pharmacokinetics (8).

Second-Generation ADCs

In general, there are three major approaches to developing second-generation ADCs. In the first method, an unnatural amino acid (uAA) is introduced into native proteins to achieve site-specific conjugation. Nature uses 20 amino acids as building blocks for proteins. To overcome the problem of non-specificity given by conjugating to natural amino acids, a 21st amino acid is added to proteins in a site-specific manner. This exogenous uAA functions as a chemical handle for the conjugation. Introduction of this 21st amino acid can be achieved through a cell-based approach involving 'nonsense' genetic codons – often the Amber stop codon UAG – to synthesise 'sense' products (proteins); it is also a technique that utilises an expanded genetic code (9,10). Although uAAs can be incorporated into antibodies with an *Escherichia coli* lysate-based cell-free protein synthesis, the antibodies produced from this system do not have glycosylation (11). The cell-free system is also not yet compatible with mainstream manufacturing facilities.

Another approach utilises the engineered cysteine for conjugation. Among several methods used, THIOMAB drug conjugates (TDC) are a representative example (12). In TDC, an exogenous cysteine is introduced via site-directed mutagenesis to an antibody. This specific cysteine is masked by conjugation to glutathione before being exposed to a reducing solvent agent. Endogenous cysteines can be reoxidised by $CuSO_4$, leaving the exogenous cysteine to be modified by the maleimide conjugation. This method has achieved highly precise reactivity in site-specific ADC products and has been shown to be rigorous enough to be used in a Phase 1 study (13). Additionally, while the stability of maleimide conjugates in the bloodstream is a concern, it has recently been shown that, at certain sites with an optimal microenvironment on the antibody surface, the maleimide conjugates can be stabilised (14).

The third site-specific ADC process involves enzymatic conjugation, which requires the insertion of peptide tags into antibody sequences. A representative method is transglutaminase-mediated conjugation as this has the ability to modify position-specific glutamines (15). A glutamine tag therefore needs to be incorporated into antibodies to render final site-specific ADCs via lysine-containing linkers (16). When 90 locations on an antibody were tested for incorporating an LLQG tag, 12 out of 90 were found to have higher levels of conjugation. Among these 12 sites, two

sites located at the C-terminus of either light chain (C16-LC) or heavy chain (C16-HC) were evaluated for AcLys-VC-MMAD conjugation. In comparison to traditional non-specific ADCs (C16 ADC), both C16-HC and C16-LC showed a twofold increase in IC_{50} in an *in vitro* cell line cytotoxicity study (16).

EuCODE System

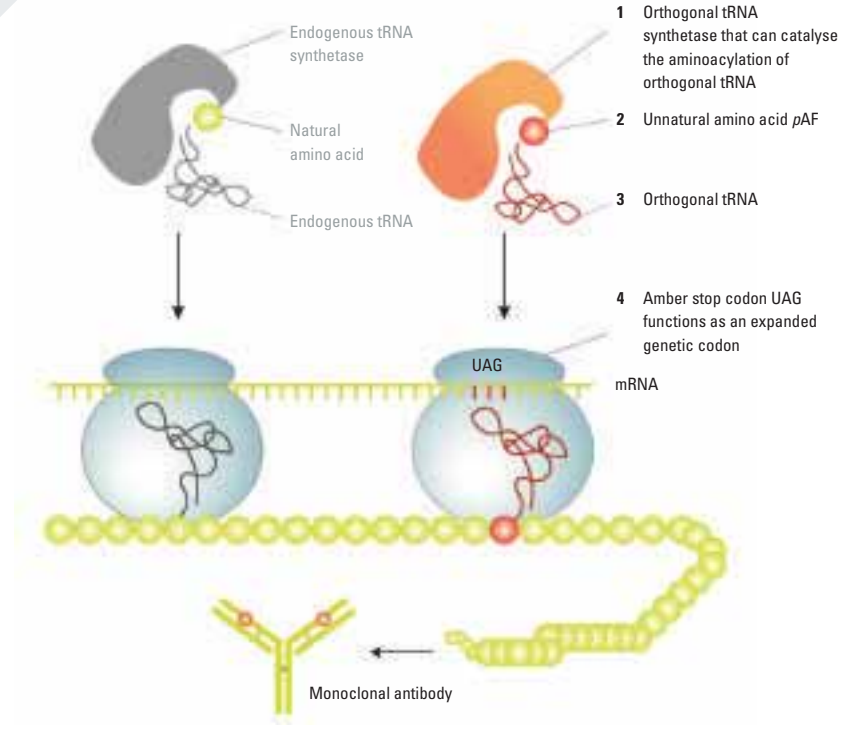
Genetically engineered ADCs use engineered bioorthogonal aminoacyl tRNA synthetase (aaRS) and tRNA to produce proteins either in *E. coli* or mammalian cells (17). The EuCODE system allows for the manufacturing of unnatural amino acid containing proteins, such as antibodies in Chinese Hamster Ovary (CHO) cells (18). There are four essential components in establishing a bioorthogonal approach that produces proteins naturally: a uAA that can be used as a handle for conjugation; an unused genetic codon that can be assigned to the UAA; a tRNA that can transport the UAA to ribosomes; and an aaRS that can catalyse the aminoacylation of the tRNA with the UAA.

As shown in Figure 1, in establishing a EuCODE system in CHO cells, the essential components are:

- Orthogonal tyrosyl-RS that can catalyse the aminoacylation of $tRNA_{Tyr}$ with the UAA *p*-acetylphenylalanine (*p*AF)
- *p*AF as a uAA with a ketone functional group that can form a specific conjugation with hydroxylamine
- *E. coli* $tRNA_{Tyr}$ that can transfer *p*AF to ribosomes
- Amber stop codon that can be used as a 'non-stop' codon for the uAA (18)

All four components have to work in concert to create a stable mammalian cell line to produce proteins, such as antibodies. Currently, an EuCODE mammalian protein expression system in CHO cells can produce unnatural amino acid-containing antibodies with titres over 1g/L. *p*AF incorporated antibody can be used for further conjugation with cytotoxic drug to create antibody drug conjugate (see Figure 2). With an anti-HER2 ADC programme advancing to clinical trials, this technology is demonstrated to be feasible for industrial manufacturing (18).

Figure 1: EuCODE system components in expressing antibodies with incorporated unnatural amino acid *p*AF



Site-specific ADCs provide homogenous components for the rational optimisation of various biophysical properties, efficacies both *in vitro* and *in vivo*, and, eventually, a therapeutic index (19). For example, evidence shows that drugs conjugated to different sites on the antibody impact the ADC thermal stability differently (18). Cleavable linkers that connect drug and antibody are desirable for several highly potent cytotoxic reagents – such as calicheamicin, PBD and duocarmycin – in order to preserve drug potencies after their release from the antibody. However, a cleavable linker is generally not stable in plasma during circulation. Scientists have shown that by moving the conjugation site just to its adjacent residue on a heavy chain, from S115 to A114, an unstable cleavable linker can be converted into a highly stable cleavable linker in plasma (16). When compared to traditional ADCs and cysteine conjugated ADCs, genetically engineered site-specific anti-HER2 antibodies reveal improved *in vitro* and *in vivo* efficacy, as well as pharmacokinetics (18).

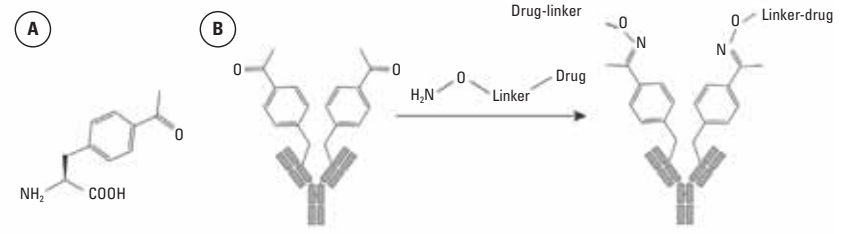
Future Direction

ADCs will continue to play an important role in the development of cancer therapies. It has been reported that cancer cells residing within an immunosuppressed microenvironment

Figure 2: ADC containing unnatural amino acid *p*AF

A Structure of *p*AF

B Antibody incorporated with *p*AF reacts to hydroxylamine linked drug to form ADC



evade immune surveillance (20). Therefore, the effects of therapeutic antibodies, bispecific antibodies and the most recent chimeric antigen receptor T cells that redirect the natural immune surveillance are compromised – especially in the case of solid tumours. However, these tumours can still be vulnerable to ADCs that deliver highly potent cytotoxic chemical drugs to targeted cancer cells.

Despite two ADC drugs already on the market, the field still faces great challenges to widening the therapeutic window. Armed with site-specific ADC technologies, scientists are well-equipped to design future-generation ADCs over the next five years with elevated potency and diminished off-target toxicity. The elevated potency can be achieved by: designing better internalising antibodies; unleashing the potency of cytotoxic drugs with cleavable linkers that are enabled by site-specific ADC technology; and designing more stable ADCs *in vivo*. At the same time, to lower off-target toxicity, researchers can: design drug linkers with the assistance of site selection to slow down off-target liver uptake *in vivo*; create differential binding antibodies that preferentially bind to the target only on cancer cells, and not the same target on normal tissue; and create cytotoxic drugs that are active only when the antibody binds to the target on cancer cells.

Site-specific ADC technologies offer a new starting point for the rational design of drugs with optimised therapeutic windows. In particular, genetically engineered ADCs are a unique system among second-generation ADCs. With an expanded genetic code, they make 'sense' protein products out of 'nonsense' genetic code. With stable mammalian cell lines, they produce proteins naturally with incorporated unnatural amino acids at industrial scale. They also provide scientists with a highly desirable drug design tool and greater design freedom. And, finally, they are well-positioned as a springboard for future-generation ADCs and targeted drug delivery.

Note

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References

1. Kaufmann SHE, Paul Ehrlich: Founder of chemotherapy, *Nature Reviews Drug Discovery* 7: p373, 2008
2. Mathe G, Tran Ba LO and Bernard J, Effect on mouse leukemia 1210 of a combination by diazo-reaction of amethopterin and gamma-globulins from hamsters inoculated with such leukemia by heterografts, *C R Hebd Seances Acad Sci* 246(10): pp1,626-1,628, 1958
3. Agarwal P and Bertozzi CR, Site-specific antibody-drug conjugates: The nexus of bioorthogonal chemistry, protein engineering and drug development, *Bioconjugate Chemistry* 26(2): pp176-192, 2015
4. Sun MMC *et al*, Alkylation strategies for the modification of specific monoclonal antibody disulfides, *Bioconjugate Chemistry* 16: pp1,282-1,290, 2005
5. Hamblett KJ *et al*, Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate, *Clin Cancer Res* 10(20): pp7,063-7,070, 2004
6. Strop P *et al*, Site-specific conjugation improves therapeutic index of antibody drug conjugates with high drug loading, *Nat Biotechnol* 33(7): pp694-696, 2015
7. Lyon RP *et al*, Reducing hydrophobicity of homogeneous antibody-drug conjugates improves pharmacokinetics and therapeutic index, *Nature Biotechnology* 33: pp733-736, 2015
8. Behrens CR and Liu B, Methods for site-specific drug conjugation to antibodies, *mAbs* 6: pp46-53, 2014
9. Hallam T, Wold ED, Wahl A and Smider VV, Antibody conjugates with unnatural amino acids, *Molecular Pharmaceutics* 12(6): pp1,848-1,862, 2015
10. Liu W, Brock A, Chen S, Chen S and Schultz PG, Genetic incorporation of unnatural amino acids into proteins in mammalian cells, *Nat Methods* 4(3): pp239-244, 2007
11. Goerke AR and Swartz JR, High-level cell-free synthesis yields of proteins containing site-specific non-natural amino acids, *Biotechnology and Bioengineering* 102: pp400-416, 2009
12. Junutula JR *et al*, Rapid identification of reactive cysteine residues for site-specific labeling of antibody-fabs, *Journal of Immunological Methods* 332: pp41-52, 2008
13. Kung Sutherland MS *et al*, SGN-CD33A: A novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML, *Blood* 122: pp1,455-1,463, 2013
14. Patterson JT *et al*, Improving the serum stability of site-specific antibody conjugates with sulfone linkers, *Bioconjug Chem* 25(8): pp1,402-1,407, 2014
15. Jeger S *et al*, Site-specific and stoichiometric modification of antibodies by bacterial transglutaminase, *Angewandte Chemie* – International Edition, 49: pp9,995-9,997, 2010
16. Strop P *et al*, Location matters: Site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates, *Chemistry and Biology* 20: pp161-167, 2013
17. Axup JY *et al*, Synthesis of site-specific antibody-drug conjugates using unnatural amino acids, *Proceedings of the National Academy of Sciences* 109: pp16,101-16,106, 2012
18. Tian F *et al*, A general approach to site-specific antibody drug conjugates, *Proceedings of the National Academy of Sciences* 111: pp1,766-1,771, 2014
19. Perez HL *et al*, Antibody-drug conjugates: Current status and future directions, *Drug Discovery Today* 19: pp869-881, 2014
20. Seliger B, Strategies of tumor immune evasion, *BioDrugs* 19(6): pp347-354, 2005

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