Genetically Engineered ADCs

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.

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 CuSO4, 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

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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 IC50 in an in vitro cell line cytotoxicity study (16).

EuCODE System

Genetically engineered ADCs use engineered bioorthogonal aminocyl 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 aminocacylation 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-aaRS that can catalyse the aminocacylation of tRNATyr with the UAA p-acetylphenylalanine (pAF)
- pAF as a uAA with a ketone functional group that can form a specific conjugation with hydroxylamine
- E. coli tRNATyr that can transfer pAF 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. pAF 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).

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
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.

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