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Therapeutic applications of an expanded genetic code

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Introduction

The genetic codes of all living organisms specify the same 20 canonical amino acids. This limited repertoire significantly restricts the chemical diversity available to the protein biosynthetic machinery for the generation of proteins with a desired structure and/or function. To overcome this limitation, a handful of proteins contain the nonstandard amino acids, pyrrolysine and selenocysteine, and many proteins are modified postranslationally. Ideally, one would like to be able to augment the genetic codes of living organisms with new building blocks that can be used to create new structures and/or functions not readily available from the common twenty amino acids. To this end, methodology has been developed that allows one to cotranslationally incorporate non-canonical amino acids (ncAA) into proteins in living cells in response to nonsense or frameshift codons. This is accomplished with an orthogonal tRNA/aminoacyl tRNA synthetase pair (tRNA/aaRS) that is specific for the non-canonical amino acid of interest and does not cross-react with endogenous amino acids, tRNAs or aaRSs.

A variety of tRNA/aaRS pairs have been evolved to genetically encode over 100 non-canonical amino acids with novel chemical or biological properties in bacteria, yeast, multicellular organisms, plants, and most recently in mammalian systems *in vitro*.^[1–5] Optimization of the tRNA and aaRS, as well as modifications to the endogenous translational machinery itself, have greatly improved the efficiency of ncAA incorporation such that therapeutic proteins have been produced with high translational fidelity in yields of up to 5 grams/L in 50,000 L bacterial fermentation reactions and at greater than 1 gram/L in stable mammalian cell lines.^[6–8] Moreover, it has been shown that multiple distinct ncAAs can be incorporated into a single protein using mutually orthogonal tRNA/aaRS pairs and

their cognate 3 and 4-base codons.^[9–11] In addition tRNA/aaRS pairs have been evolved to encode ncAAs with diverse properties including bio-orthogonal chemical conjugation partners, metal chelators, photo-crosslinking and photo-caged side chains, natural post-translational modifications, redox-reactive agents as well as IR, NMR, and fluorescent probes.^[1, 2]

This methodology has been widely used to site-specifically introduce into proteins biophysical and chemical probes of protein structure and function both *in vitro* and *in vivo*.^[12–22] It has also been used to rationally design and/or evolve proteins with new or enhanced activities.^[23–28] Importantly, this technology enables the incorporation of a ncAA at almost any site in the proteome with minimal perturbation of protein folding and function and with medicinal chemistry-like control over structure. This latter capability is being increasingly exploited for the site-specific modification of proteins with drugs, polyethylene glycols (PEGs), nucleic acids and imaging agents for therapeutic or diagnostic applications.^[8, 27, 29–32] Incorporation of ncAAs with bio-orthogonal chemically reactive groups, as opposed to the nucleophilic side chains of cysteine and lysine, allows for the synthesis of chemically modified recombinant proteins that are structurally defined, homogeneous molecular entities rather than mixtures of proteins with distinct conjugation sites and stoichiometries.^[33–39] This ability should allow one to rationally optimize the pharmacological properties of biologics, much the same way chemists currently optimize the pharmacology of small-molecule therapeutics. Here we discuss the recent exploitation of ncAA technology to overcome existing challenges in the production of current protein therapeutics and to develop novel therapeutic modalities.^[25]

Antibody-drug conjugates

Antibody-drug conjugates (ADCs) allow one to target drugs to specific tissues to increase both their efficacy and safety (the modern version of Ehrlich's "magic bullet").^[40] The majority of tissue-associated antigens that are targeted by ADCs undergo receptor-mediated endocytosis, where upon binding, the ADC is internalized with its target antigen and the cytotoxic drug is released intracellularly by degradation of the conjugate (Figure 1A).^[41] Therefore, the efficacy of an ADC relies heavily on target specificity, receptor density on the cell surface, rate of drug internalization, and properly controlled drug release, all of which should be optimized for maximal efficacy and safety.^[42] Currently, three ADCs have been approved by the FDA: gemtuzumab ozogamicin (Mylotarg; anti-CD33 IgG linked to a calicheamicin) for acute myelogenous leukemia, brentuximab vedotin (Adcetris; anti-CD30 IgG linked to a monomethyl auristatin E) for Hodgkin's lymphoma, and trastuzumab emtansine (T-DM1 or KADCYLA; anti-HER2 IgG linked to a maytansinoid) for HER2-positive breast cancer.^[43–49] Gemtuzumab ozogamicin, however, was voluntarily withdrawn from market due to toxic side effects and inefficacy, indicating that ADC engineering could benefit from further optimization in design and validation.^[41]

Conventional antibody conjugation involves modification of cysteine or lysine side chains by electrophilic agents; however, these reactions provide only limited control over conjugation site due to the presence of multiple cysteine and lysine residues in antibodies.^[25] Site and stoichiometry of drug modification have been shown to affect ADC

stability and aggregation, blood clearance, binding affinity, and other factors affecting potency and tolerability.^[42] For example, the clinical drug T-DM1 is typically synthesized through non-specific lysine conjugation of trastuzumab, to afford an average of 3.5 DM1 per mAb.^[48] A similar ADC has been engineered by Genentech using site-specific THIOMAB technology, where cysteine residues in the heavy-chain constant region of a trastuzumab mutant were introduced as specific conjugation handles to attach precisely 2 DM1 molecules.^[50] Toxicology studies indicated that the homogeneous THIOMAB version was better tolerated in both rodent and non-human primate models than the randomly conjugated T-DM1.^[51] Similarly, Seattle Genetics developed a site-specific conjugation strategy in which a residue of the anti-CD70 heavy chain was mutated to a solvent-exposed cysteine that was subsequently modified with the DNA cross-linker pyrrolobenzodiazepine (PBD). The site-specific anti-CD70-PBD showed better drug uniformity as well as lower levels of aggregation compared to random conjugates.^[52] Despite these advances, both approaches involve uncapping of unpaired sulfhydryl groups, which requires additional steps in their conjugation and purification protocols, due in part to the formation of multimeric side products.^[53] Furthermore, many cysteine adducts are less stable under physiological conditions, which can limit their utility in therapeutic applications. An optimal conjugation handle would be one that can be site-specifically introduced at virtually any desired surface site in a protein with minimal perturbation to protein structure, have chemical reactivity that is orthogonal to the side chains of native proteins, and also result in a physiologically stable bond.^[54] Among the many methods being explored to introduce new functionalities in therapeutic proteins include chemoenzymatic reactions, such as the use of formylglycine-generating enzyme (FGE) to convert cysteine to an aldehyde handle, a transglutaminase method to attach drug payloads to glutamine tags, and glycoengineering strategies.^[55–57] Genetic incorporation of selenocysteine as well as auxotrophic introduction of ncAAs have also been explored in *Escherichia coli* (*E. coli*) systems.^[58]

The use of genetically encoded ncAAs as unique chemical handles affords a straightforward strategy to introduce a uniquely reactive amino acid side chain at one or more desired sites in a protein by simple recombinant methods. This approach was exemplified by the site-specific incorporation of *p*-acetyl-phenylalanine (pAcF) into the antigen-binding fragment (Fab) of an antibody using a *Methanococcus jannaschii* (*Mj*)-derived tRNA/RS pair in *E. coli*.^[33] Expression levels of recombinant protein mutants containing pAcF were comparable to those of wild-type protein. The ketone moiety of pAcF allows for the selective formation of an oxime bond with an aminooxy-derivatized drug.^[25] Reaction of the mutant antibody and drug at pH 4–5 affords homogeneous conjugates in almost quantitative yields. The oxime bond is stable under physiological conditions, and the structural similarities of pAcF to the natural amino acids tyrosine and phenylalanine allow for minimal structural perturbations to the protein. The first application of this approach involved the HER2-targeting antibody, Herceptin (trade name for trastuzumab).^[40] Full-length IgG Herceptin containing two pAcF residues was expressed in mammalian cells by transient transfection with an expression vector encoding the amber nonsense mutant of the antibody gene as well as an *E. coli*-derived tRNA/RS pair specific for pAcF.^[29, 60] This Herceptin mutant was then conjugated to an auristatin analogue with a terminal aminooxy group with > 90% yield and > 95% purity. The final ADC product showed excellent *in vitro*

cytotoxicity against HER2-positive breast cancer cells (HER2-transfected MDA-MB-435 and SK-BR-3 cell lines) with EC₅₀ values of 100–400 pM. Furthermore, complete tumor regression in rodent xenograft models bearing HER2-positive solid tumors was also observed within 14 days after initiation of treatment without overt toxicity. In addition, the ADC was found to have a serum half-life in rodents similar to Herceptin itself, in contrast to nonspecific conjugates that typically have significantly reduced half-lives. To produce ncAA-containing mutant IgGs at a practical scale for commercial production, Tian and co-workers developed a platform cell line in which the engineered tRNA/RS pair was stably integrated into the genome of Chinese hamster ovary (CHO) cells.^[8] Introduction of amber mutants of Herceptin afforded the corresponding pAcF Herceptin mutants at high titers of > 1 gram/L in a fed-batch bioreactor. The generality of this process was demonstrated through the establishment of other stable cell lines expressing anti-5T4, anti-EGFR and anti-PSMA antibodies in similar yields.

A site-specific Herceptin-monomethyl auristatin D (MMAD) ADC (ARX788) generated by the above methods was compared in a number of preclinical studies with a conventional non-specifically conjugated ADC (synthesized through reaction of a maleimide-modified linker with reduced interchain disulfide bonds; average DAR ~ 4.5;) and site-specific THIOMAB (DAR ~ 2.3).^[54] *In vitro* cytotoxicity assays using cancer cell lines expressing HER2 at varying levels showed that ARX788 demonstrated superior *in vitro* activity (higher potency against HER2-positive cells but less toxicity against HER2-negative cells) in comparison to the conventional ADC, despite the lower DAR of the site-specific conjugate. It is noteworthy that the ncAA-generated site-specific ADC showed better efficacy relative to the other format ADCs even in the presence of cell lines expressing low levels of antigen (2+). This same superior activity of ncAA-based site-specific ADCs was also observed in *in vivo* efficacy studies, where the ADC eliminated established solid tumors at 1 mg/kg dose in two xenograft models with an improved therapeutic index. In comparative pharmacokinetic studies using anti-5T4 antibodies as a model system, the ncAA-based ADC showed similar systemic exposure to wild-type antibody and a significantly slower clearance rate from serum in comparison to the conventional ADC with higher DAR. Recently, a more in-depth comparative study was conducted, which further demonstrated the favourable pharmacokinetics and serum stability of ncAA-based site-specific ADCs over conventional ADCs.^[54] For example, after 28-day incubation with human serum, the conventionally cysteine-conjugated ADC sample showed a decrease of 36% in total antibody concentration, of which 55% of the remaining antibodies were still conjugated. The site-specific conjugates, in contrast, remained intact for the entire time period and experienced only a 25% decrease in total antibody concentration; notably, payload loss was observed with the THIOMAB anti-MUC16-monomethyl auristatin E (MMAE), which also relies on a cysteine-based maleimide reaction, after only 4 days in serum.^[61] The methodology described above has similarly been used to generate anti-CXCR4, anti-PSMA and anti-CD74 antibodies conjugated to toxins for the treatment of metastatic cancer, prostate cancer, and renal cancer, respectively. In most of these studies it was shown that conjugation site can significantly improve the efficacy and safety profile for a therapeutic ADC, underscoring the value of synthesizing and optimizing chemically defined, homogeneous protein conjugates.

These previous studies relied on Fab expression in *E. coli* or IgG expression in mammalian cell culture, but more recently, an open cell-free synthesis (OCFS) system for protein expression has been optimized for the production of proteins harbouring ncAAs.^[62] Aglycosylated full-length Herceptin IgG containing *p*-azidomethyl phenylalanine (pAzMF) was expressed using OCFS and conjugated to dibenzocyclooctyl (DBCO)-PEG-MMAE. This site-specific Herceptin-MMAE conjugate also demonstrated potent and selective *in vitro* activity. The copper-free [3 + 2] Huisgen cycloaddition (or “click”) reaction between an azide and cyclooctyne can be carried out at neutral pH, and the resulting 1,2,3-triazole bond is also physiologically stable.^[63] Whether this methodology can be scaled for commercial production remains to be seen.

These examples of site-specific ADC synthesis illustrate the advantages of ncAA-mediated conjugation with respect to control over the site and stoichiometry of conjugation, homogeneity of the drug substance, and *in vitro* and *in vivo* preclinical pharmacology. The recent expansion of the genetic toolbox to include ncAAs with additional reactive moieties will further improve conjugation efficiency and diversify the structures of toxins and linkers that can be exploited in future ADC synthesis.^[35–37] Indeed, we have recently successfully prepared ADCs that consist of a liver-specific antibody conjugated to a thyroxine derivative to selectively deliver thyroid hormone receptor (TR) agonists to the liver for the treatment of nonalcoholic steatohepatitis (NASH), the kinase inhibitor dasatinib conjugated to a T-cell targeting antibody for the selective delivery of immunosuppressants to lymphocytes, and inhibitors of epigenetic modifications for cancer therapy.

Bispecific antibodies for cancer immunotherapy

Cytotoxic T cells recognize their targets through an interaction between the T-cell receptor (TCR) and its cognate major histocompatibility complex (MHC) class I molecule presented on target cells. The formation of an “immunological synapse” triggers TCR signalling, which plays a crucial role in T-cell activation and the expression of cytotoxins and other signalling molecules to induce apoptosis in the target cell. It has been demonstrated that a bispecific antibody that simultaneously binds to the CD3 protein, a component of the TCR complex on T cells, and a tumor-associated antigen on cancer cells can also induce TCR signalling in a similar (but MHC-independent) fashion by crosslinking multiple TCR complexes, leading to specific target cell lysis (Figure 1B).^[64] As MHC downregulation is one of putative mechanisms of how cancer cells escape normal immune surveillance by T cells, bispecific antibodies offer a feasible strategy for subverting these evasive features by directly recruiting immune effectors to the tumor cell. Furthermore, this bispecific approach is not susceptible to the mechanisms that lead to multidrug resistance in tumors, such as upregulation of drug transporters. In addition, bispecific antibodies are likely to be effective against quiescent cancer stem cells and against cells expressing a low-density of the targeted antigen since they do not require receptor-mediated internalization, a significant advantage over ADCs. Indeed, two bispecific antibodies, catumaxomab (anti-EpCAM/anti-CD3) and blinatumomab (anti-CD19/anti-CD3), have shown impressive results for the treatment of malignant ascites and refractory acute lymphoblastic leukemia (ALL), respectively, and several other bispecific antibodies are generating promising results in preclinical and early clinical stages.^[64]

Traditional methods for generating bispecific antibodies have largely relied on genetic fusions, which limits the spatial arrangements available to the two ligand-binding domains.^[64–68] The formation of an effective “pseudo-immunological synapse,” depends on the relative orientation and distance between each component of a bispecific molecule.^[69, 70] Additionally, engineered scaffolds, especially those based on scFvs (single-chain variable fragments), often suffer from low expression yields, aggregation (leading to nonspecific T-cell activation), and poor half-lives (requiring continuous infusion).^[71] As an alternative, bispecific antibodies can be generated by semi-synthetic means, where each mAb or binding domain is conjugated through chemical methods, allowing for more control over geometry and distance. There are some examples of semi-synthetic bispecific antibodies using cysteine and lysine conjugation chemistry, but these reactions result in complicated and heterogeneous products, as was also observed in ADC synthesis. If any of these products are unstable and aggregate, they may lead to non-specific T-cell activation due to CD3 crosslinking that could potentially induce undesirable cytokine release and reduce drug safety.^[72]

To overcome these shortcomings, ncAAs with orthogonal chemical reactivity have been used to generate bispecific antibodies by site-specific conjugation of the two ligand-binding domains using flexible PEG linkers. This approach affords chemically defined, homogeneous bispecific antibodies with a defined orientation and distance between the binding domains. To exemplify this approach, a facile two-step conjugation reaction was employed to synthesize an anti-HER2/anti-CD3 Fab heterodimer. pAcF was incorporated into the constant regions of anti-HER2 and anti-CD3 Fabs, and then each was separately conjugated to a flexible PEG linker functionalized with an aminooxy moiety on one end and either an azide or cyclooctyne reactive group at the other terminus. These two domains were then conjugated through a copper-free “click” reaction; the conjugation efficiency was 70% as determined by SDS-PAGE and chromatographic separation, and the heterodimer was easily purified from unreacted Fabs by size-exclusion column. The resulting anti-HER2/anti-CD3 Fab bispecific antibody effectively crosslinked HER2-positive cancer cells to CD3-positive T-cells *in vitro*; in an *in vitro* cytotoxicity assay, where human peripheral blood mononuclear cells (hPBMCs) were mixed with HER2-transfected MDA-MB-435 cells at a ratio of 10:1, the conjugate demonstrated cytotoxicity at picomolar concentrations ($EC_{50} \sim 20$ pM). Cytotoxicity was not observed when an unconjugated mixture was used or when HER2-negative MDA-MB-435 cells were treated, indicating that the anti-HER2/anti-CD3 conjugate mediated T-cell-induced apoptosis only in HER2-positive cancer cells. Given that the currently approved anti-HER2 mAbs and ADC are only offered to breast cancer patients with high levels of HER2 expression, the anti-HER2/anti-CD3 bispecific approach may benefit patients with low HER2 expression levels (scored 1+ and 2+ by immunohistochemistry), who form a majority of HER2-positive breast cancer patients (up to 40–50%).^[73–75]

A more versatile laboratory-scale Fab ligation strategy has been demonstrated using highly selective Watson-Crick base pairing interactions to selectively couple the ligand-binding domains. In particular, an anti-HER2 Fab with pAcF was site-specifically conjugated with an aminooxy-oligonucleotide linker.^[76] Separate anti-HER2 Fabs were modified with

complementary oligonucleotide sequences, and upon mixing of these two conjugates, anti-HER2 Fab homodimeric bispecific antibodies were rapidly formed in almost quantitative yields. Activity was confirmed in a HER2 *in vitro* phosphorylation assay, and it was noted that a cleavable linker could also be formed by introducing an internal nuclease cleavage sequence. Similarly, PNA (peptide nucleic acid)-based linkers were generated to conjugate either an anti-HER2 or anti-CD20 Fab to an anti-CD3 Fab to afford a heterodimeric bispecific antibody. Due to the higher melting temperature of PNAs, the stability of these linkers allowed them to survive purification through conventional chromatography; additionally, the structures of PNAs are resistant to degradation by both serum nucleases and proteases.^[77] These PNA-linked anti-HER2/anti-CD3 and anti-CD20/anti-CD3 displayed excellent activity in recruiting T lymphocytes to HER2-positive breast cancer cells and CD20-positive B-cell leukemia cell lines, respectively, *in vitro*. The additional advantage of oligonucleotide-based linkers is in the formation of antibody multimers with more complex architectures; for example, an anti-CD20 tetramer was generated using orthogonal PNAs and induced apoptosis in CD20-positive Ramos cells in a dose-dependent manner^[76]. This methodology should allow one to rapidly survey the biological activity of different combinations of binding domains *in vitro* against different target tumors.

Bispecific molecules that target T cells to cancer cells have also been generated using small-molecule ligands, rather than an antibody fragment, to selectively bind receptors on the cancer cell surface (Figure 1C). This approach was first used to target T cells to prostate cancer which often progresses to hormone-refractory prostate cancer (HRPC) with a 1–2 year median survival prognosis. Currently, there is no effective therapy for HRPC, and the first-in-class ADC (PSMA ADC, Progenics) for this disease recently showed significant hematologic toxicity in Phase I clinical trials, which likely caused the fatal infections reported in early Phase II studies.^[78, 79] Again, a bispecific antibody approach may afford a more efficacious alternative to conventional drug-based strategies (e.g., ADCs and cytotoxic agents), as T cells may overcome many of the mechanisms that render cytotoxic drugs less effective.

Although its role in prostate cancer pathogenesis is unclear, prostate-specific membrane antigen (PSMA), a zinc-dependent metallopeptidase, is homogeneously and highly expressed on the cell surface of prostate cells during all stages of prostate cancer, but only minimally present on the surface of other tissue types, making it an ideal target for immunotherapy. Previously, a series of natural substrate analogues (*N*-acetyl-aspartyl-glutamate, NAAG) have been developed for the inhibition of PSMA enzymatic activity. Among them, 2-[3-(1,3-dicarboxy propyl)-ureido] pentanedioic acid (DUPA), binds tightly ($K_i = 8$ nM) to PSMA and has been previously used as a targeting moiety in imaging and toxin delivery for prostate cancer.^[80] In contrast to antibodies, such small-molecule-based targeting agents are amenable to extensive chemical modification to improve affinity, selectivity, and pharmacokinetics and can also be easily conjugated to T-cell-specific antibodies.^[81] To generate a PSMA-targeting small molecule-antibody conjugate (SMAC), we carried out a structure-activity study, which yielded a very high affinity ($K_i = 2.5$ nM) DUPA-linker conjugate.^[82] To form a site-specific DUPA-antibody construct, an anti-CD3 Fab was expressed with pAcF in *E. coli* and conjugated to the DUPA linker compound

through a one-step oxime bond reaction, similarly to that used in ADC synthesis (Scheme 1).^[81] Binding studies using CD3-positive cell lines demonstrated uniform binding across different DUPA-linker conjugates, indicating that conjugation did not compromise the binding affinity of the anti-CD3 antibody. In contrast, binding to PSMA-positive cell lines did vary based on either the affinity of the DUPA-linker compound or linker length. When the same DUPA-linker compound was conjugated at different positions in the anti-CD3 Fab, binding to neither T cells nor PSMA-positive cells was significantly affected. However, interestingly, when the conjugates were evaluated in an *in vitro* cytotoxicity assay, their abilities to produce an effective pseudo-immunological synapse differed significantly. This result highlights an advantage of the semi-synthetic approach in providing more control over the spatial orientation of bispecific antibodies, which is not so easily achieved through genetic fusion. The incorporation of two pAcFs into anti-CD3 (in both the heavy and light chains) was also achieved without compromising expression yields and enabled the synthesis of a bivalent DUPA conjugate to enhance target cell binding via bivalent binding. The optimized PSMA-targeting bispecific SMAC (P-SMAC) showed potent *in vitro* killing activity (EC₅₀ ~ 100 pM) against PSMA-positive prostate cancer cells (but not PSMA-negative cells) in the presence of human T lymphocytes. Significantly, this conjugate also demonstrated potent anti-tumor activity in rodent xenograft models with no overt toxicity in immunodeficient mice that were engrafted with human PBMCs. P-SMAC also has an improved serum half-life in rodents (t_{1/2} ~ 5 hours) relative to the unconjugated Fab (t_{1/2} ~ 1 hour) despite their similar molecular weights (~ 50 kDa). The excellent preclinical activity of P-SMAC demonstrates the utility of ncAA technology in providing exquisite control over the geometry and orientation of semi-synthetic bispecific immunotherapeutics to optimize formation of the immunological synapse.

Another SMAC was recently generated to recruit cytotoxic T cells to folate receptor (FR)-positive cells. FR is known to be overexpressed on the surface of inflammatory cells as well as many types of cancer cells, such as ovarian and non-small-cell lung cancers. Folic acid (vitamin B₉) selectively binds FR with high affinity ($K_d < 1$ nM) and has also been extensively used as a carrier molecule in FR-targeted delivery of chemotherapeutic drugs and imaging agents.^[83] To synthesize a site-specific FR-targeting SMAC, a solvent-exposed γ -carboxylic acid group in folic acid was modified with a PEG linker, and the folate-PEG-aminoxy ligand was then conjugated to an anti-CD3-pAcF mutant Fab.^[84] Again, flow cytometry-based experiments confirmed that conjugation did not alter the native binding of folic acid or the anti-CD3 Fab. The anti-CD3 Fab-folate conjugate demonstrated potent killing of FR-positive cancer cells in the presence of human PBMCs, while FR-negative cells were unaffected even at higher concentrations of the SMAC. *In vivo* xenograft studies also showed that human PBMC-engrafted mice can effectively eliminate FR-positive tumor burden after treatment with the conjugate. Notably, post-experimental histopathological analysis showed no difference between treated and non-treated groups, suggesting a favourable therapeutic index for the FR-targeting SMAC. The promising preclinical data from these SMACs indicates that ncAA methodology need not be limited to the conjugation of proteins to other proteins but can also be extended to tumor-specific small-molecule ligands of diverse structures and specificities. Indeed, we are currently exploring other such

ligands, including peptides (e.g., gonadotropin-releasing hormone and gastrin derivatives) and additional small molecules.

Antibody-polymer conjugates for siRNA delivery

The usefulness of ncAAs for forming site-specific antibody conjugates has also been extended to tissue-specific anti-sense oligonucleotide (ASO) and siRNA delivery. The ability of ASOs and siRNAs to selectively silence gene expression has inspired the ongoing development of oligonucleotide-based therapeutics to treat a variety of human diseases.^[85] However, a significant obstacle to any oligonucleotide-based therapy is the lack of efficient delivery mechanisms. Due to the size and hydrophilicity of siRNAs, it is necessary to engineer delivery agents that can transport the siRNA to its desired intracellular target without risking degradation or clearance from the bloodstream.^[86]

Recently, ncAA-directed conjugation was employed to generate an antibody-polymer conjugate (APC) that effectively targeted HER2-positive breast cancer cells (Figure 1D).^[32] The side-chain guanidinium groups of the cationic polymer reversibly bind siRNA while allowing its release within the endosome or lysosome.^[87, 88] Anti-HER2 Fabs and IgGs were expressed with pAcF at various sites and then conjugated to an aminoxy-derivatized cationic polymer, P1. Interestingly, A121-IgG-P1 was ineffective in siRNA delivery, whereas siRNA delivery was demonstrated with anti-HER2 S202-Fab-P1 and Q389-IgG-P1 (using FITC-labelled siRNA), again emphasizing the influence of site selection on the efficacy of the final conjugation product. The S202-Fab-P1 and Q389-IgG-P1 anti-HER2 conjugates bound HER2-positive cancer cells with no detectable cytotoxicity. Delivery of siRNA by the antibody-P1/siRNA complex (100 nM) effectively silenced expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at the RNA and protein expression levels; no GAPDH gene silencing was observed in HER2-negative cell lines. Further improvements in potency may be required, and *in vivo* activity has not yet been demonstrated. Nonetheless, the utility of ncAA technology in generating antibody conjugates shows promise for a wide variety of therapeutics in cancer therapy.

Increasing therapeutic protein serum half-life

One common method to improve protein therapeutics that suffer from poor pharmacokinetics is the conjugation of a high molecular-weight synthetic polymer, such as PEG, to decrease renal clearance and potentially protect from proteolysis. Indeed, the conjugation of PEG to a variety of therapeutic proteins has increased their stability and serum half-life, thereby improving efficacy and decreasing dose frequency *in vivo*.^[89] The bulky size of the PEG polymer is likely to significantly impair ligand-receptor binding if randomly conjugated, and again each distinct conjugate in a heterogeneous mixture is likely to differ in efficacy and safety, making ncAA-mediated site-specific conjugation an attractive strategy. Indeed, the ability to define a distinct location for PEG conjugation has proven crucial in the design and synthesis of a human growth hormone (hGH) analogue. One of the established therapies for hGH deficiency-associated growth defects is subcutaneous injection of hGH. However, the short circulating half-life of recombinant hGH (20–30 minutes) necessitates daily injection. Incorporation of pAcF into hGH allowed for the optimization of the pharmacology of PEGylated hGH since, in contrast to nonspecific

conjugation, defined homogeneous conjugates could be synthesized and evaluated *in vitro* and *in vivo*. The optimized construct demonstrated similar affinity and bioactivity compared to unmodified hGH.^[27] Furthermore, the monoPEGylated conjugate showed significantly improved pharmacokinetics with no overt toxicity in rat models, which enabled weekly injection in clinical trials with human patients. In the Phase 2 clinical trials, the site-specific monoPEGylated hGH (ARX201) was also well tolerated, exhibiting higher potency and requiring reduced injection frequency relative to standard hGH therapy.

In addition to ARX201, several other PEG-conjugated hormones generated using ncAA technology have been reported. For example, PEGylated human fibroblast growth factor 21 (hFGF21) and leptin have been developed for the treatment of type II diabetes and obesity, respectively.^[30] Again, in both cases, site-specific pAcF incorporation (and thus PEG conjugation) had a significant impact on the resulting *in vitro* and *in vivo* function of the conjugates. hFGF21-PEG demonstrated potent glucose-lowering activity without reducing body weight or producing hypoglycemia in *db/db* mouse models. The reduced incidence of hyperglycemia appeared to result from increased energy expenditure as well as improved pancreatic function and insulin sensitivity in all treated mice. The optimized PEGylated hFGF21 (ARX618) is currently undergoing Phase 2 human clinical trials. In total, these therapeutic protein-PEG conjugates demonstrate the potential of ncAA methodology in optimizing the pharmacokinetic properties of existing biologic drugs to treat or manage chronic illnesses.

Therapeutic vaccines using an expanded genetic code

Another potential therapeutic application of ncAA technology is in the generation of therapeutic vaccines. This was first demonstrated by the use of the ncAA *p*-nitrophenylalanine (pNO₂F) to break immunological tolerance to self-proteins in rodents and generate an autologous antibody response. Nitroaryl groups have previously been used as immunogenic haptens, and clinical trials have suggested the utility of dinitrophenyl-modified cancer cells as a vaccine for melanoma.^[90] pNO₂F was incorporated into a murine inflammatory cytokine, tumor necrosis factor- α (mTNF- α), at position Y86, where the mutation results in a negligible change in protein folding/stability but was expected to generate a new CD4 T-cell neo-epitope.^[23] Injection of the pNO₂F86 mTNF- α mutant induced a robust antibody response to both the mutant and native mTNF- α in mouse models, even in the absence of adjuvant, while wild-type mTNF- α did not. Follow-up mechanistic studies revealed that this mutation indeed generates a T-cell neo-epitope that enhances a productive interaction between CD4 T and autoreactive B cells.^[91] To validate the immunogenic contribution of the nitroaryl group, a Y85F mTNF- α mutant was also immunized, and no measurable immune response was mounted. The polyclonal antibody response generated against the pNO₂F mutant effectively protected mice against endotoxemia induced by a lipopolysaccharide (LPS) challenge. Similar results were found by substituting pNO₂F at D42 and K11, but not at F104 or F19, reinforcing the importance of ncAA context dependence. The pNO₂F86 mTNF- α mutant was shown to be effective in inducing persistent immunity, with polyclonal IgG antibody levels maintained at > 80% of their initial levels up to 40 weeks post-injection.^[92] Similar studies were performed with complement component 5 α (C5 α) and retinol-binding protein, and are ongoing with PCSK9

(proprotein convertase subtilisin/kexin type 9). Moreover, it has been shown that natural posttranslational modifications (e.g., nitrotyrosine and sulfotyrosine) can also break tolerance when introduced at specific sites in proteins.^[91] This observation supports the notion that natural autoimmune disease may be initiated by aberrant posttranslational modifications to endogenous proteins resulting from viral or other cellular stress. We are currently attempting to use a similar strategy to increase the immunogenicity of protein vaccines against foreign pathogens.

Vaccines comprised of whole attenuated microorganisms generally provide more effective immune protection against a disease-causing agent than subunit vaccines (i.e., those that contain some, but not all, of a pathogen's components).^[93] However, the potential infectivity of whole attenuated microbes hinders attempts to find suitable vaccines for highly infectious agents, such as malaria, methicillin-resistant *Staphylococcus aureus* (MRSA), and tuberculosis (TB). Recently, the *MjTyr* tRNA/RS pair was adapted to successfully incorporate multiple ncAAs in *Mycobacterium smegmatis*, *Bacillus Calmette-Guérin* (BCG), and *Mycobacterium tuberculosis* (*Mtb*).^[94] In addition, several pyrrolysine analogues were recently incorporated into the surface proteins of live and intact human hepatitis D virus (HDV), a satellite virus of hepatitis B (HBV), which was expressed in the human hepatocyte cell line Huh-7.^[95] ncAA incorporation into essential proteins of a pathogenic organism (in particular, two distinct proteins with two different nonsense or frameshift codons) is expected to make that organism dependent on the presence of the ncAA for proliferation. Injection of an auxotrophic pathogen grown in the presence of the ncAA would provide a robust response against the native pathogen, but the latter would be eliminated from the host upon depletion of the ncAA. If successful, pathogenic microbes might be administered with a greatly reduced risk for infectivity due to their ncAA dependence, opening up new avenues for vaccine therapy for infectious disease.

Outlook

The ability to add new building blocks to the genetic codes of living organisms has allowed us to create powerful new probes of protein structure and function, both *in vitro* and *in vivo*, as well as generate proteins with new or enhanced properties. Importantly, this technology allows medicinal chemistry-like precision in our ability to modify the structures and properties of proteins. This capability has been largely lacking in the production of most chemically modified therapeutic proteins to date, resulting in the use of heterogeneous drug substances with components that likely differ in efficacy and safety. On the basis of the promising preclinical and clinical examples described above, we are optimistic that an expanded genetic code will lead to a new generation of chemically-defined protein therapeutics with improved pharmacology. In addition, one can envisage other therapeutic applications of ncAA technology. For example, adaptation of the methodology to *Streptomyces* may allow the rational engineering of ribosomally synthesized natural products with improved antibiotic or anticancer activities. In addition, we have shown that one can genetically introduce amino acids with "chemical warheads" (e.g., vinyl sulfonamides, boronates, and reactive ketones) site-specifically into proteins to potentially generate highly selective irreversible antibody-based inhibitors of serum proteases or other extracellular or membrane-bound proteins.^[24] In conclusion, the ability to genetically

encode additional amino acids with novel structures and functions is beginning to allow us to optimize protein therapeutics with the same sort of chemical control that has historically been applied to small molecule therapeutics.

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List of abbreviations

aaRS	aminoacyl tRNA synthetase
ADC	antibody-drug conjugate
CD3	cluster of differentiation 3
CHO	Chinese hamster ovary
DAR	drug-to-antibody ratio
DUPA	2-[3-(1,3-dicarboxypropyl)-ureido] pentanedioic acid
Fab	antigen-binding fragment
FGF21	fibroblast growth factor 21
FR	folate receptor
HER2	human epidermal growth factor receptor 2
HRPC	hormone-refractory prostate cancer
hGH	human growth hormone
IgG	immunoglobulin G
mAb	monoclonal antibody
MHC	major histocompatibility complex
ncAA	non-canonical amino acid
pAcF	<i>para</i> -acetyl-L-phenylalanine
PBMC	peripheral blood mononuclear cell
PEG	polyethylene glycol
PNA	peptide nucleic acid
PSMA	prostate-specific membrane antigen
scFv	single-chain variable fragment
SMAC	small-molecule antibody conjugate
T-DM1	trastuzumab emtansine
TCR	T-cell receptor

TNF- α tumor necrosis factor α

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Figure 1.

Overview of antibody conjugates synthesized through ncAA-mediated conjugation: (A) antibody-drug conjugates (ADCs) to deliver cytotoxic drugs, (B) bispecific antibodies and (C) small-molecule drug conjugates (SMACs) to recruit cytotoxic T cells, and (D) antibody-polymer conjugates for siRNA delivery,

anti-CD3 Fab-pAcF mutant

DUPA-linker

Scheme 1.

Synthesis of a PSMA-targeting small-molecule antibody conjugate (SMAC) through oxime-bond formation between an anti-CD3 pAcF-mutant and an aminoxy-derivatized DUPA ligand