Perspective

CellPress

Human Therapies as a Successful Liaison between Chemistry and Biology

Patrick A. Baeuerle[1] and Jerry A. Murry[2,*]
[1]Amgen Research (Munich) GmbH, Staffelseestrasse 2, 81477 Munich, Germany
[2]Amgen, Inc., Process Development, Thousand Oaks, CA 91320, USA
*Correspondence: jmurry@amgen.com
http://dx.doi.org/10.1016/j.chembiol.2014.08.014

The development of novel therapies is arguably one of the most important roles of modern chemistry and biology. Here, we shed light on a particular class of human therapies in which synthetic chemical entities are attached to expressed biologicals (proteins) with the goal to enhance clinical activity. We focus our discussion on three key categories of these derivatized biologicals: proteins conjugated with biologically inert molecules, proteins conjugated with biologically active small molecules and peptides (e.g., antibody drug conjugates [ADCs]), and proteins conjugated with radio isotopes. Overall, it is apparent by the impact on clinical activity as well as the commercial success that chemical modification of recombinant proteins is becoming of increasing importance. Therefore, we argue that deeper understanding of the chemical reactions between large proteinaceous molecules and small molecule reagents will allow for more precise and elegant solutions to existing limitations in this field.

Human therapeutics are divided into small molecules ($<10^3$ Da molecular weight) and large molecules; cell-derived biological drugs, biologics (mostly proteins), have molecular weights ranging from 10^4 to 10^6 Da. Traditionally, they have been considered to fall under the purview of two separate industries—the pharmaceutical, with interest in small molecules, and biotechnology, with interests in biologics—as their discovery and development paths use fundamentally different techniques. Small molecule therapeutics are typically derived from high-throughput screening of chemical compound libraries followed by medicinal chemistry and target structure-guided lead optimization. Biological drugs are initially derived from living organisms and drug leads generated by employing biotechnology and recombinant DNA technology. The huge success of therapeutic proteins, mostly monoclonal antibodies (mAbs) and hormones, in the last two decades raised an enormous interest in biologics at pharmaceutical companies that traditionally focused on small molecule drug discovery and development. Protein-based drugs are the largest and fastest growing class of therapeutics with $165 billion (B) (of $754B total) in world-wide sales in 2013. Moreover, 6 of the 10 top-selling therapeutics in 2013 were proteins, namely, adalimumab (Humira, $11.0B), etanercept (Enbrel, $8.8B), infliximab (Remicade, $8.4B), insulin glargine recombinant (Lantus, $7.6B), rituximab (Rituxan and Mab-Thera, $7.5B), bevacizumab (Avastin, $6.8B), and trastuzumab (Herceptin, $6.6B). By 2020, it is projected that protein-based therapeutics will reach >$300B in worldwide sales, with the anti-TNF-α antibody adalilumab (Humira) as the single largest selling drug with more than $12B in annual sales in 2018 (Evaluate Pharma 2014 Outlook, http://www.evaluategroup.com/public/Reports/EvaluatePharma-World-Preview-2014.aspx). As a consequence, we have seen bold acquisitions of biotechnology companies by large pharmaceutical companies while others have made very significant investments to build their in-house biological drug divisions from scratch. Prominent examples of merger and acquisition activities related to adopting

biotechnology include Genentech by Roche ($46.8B), Imclone by Eli Lilly ($6.5B), Medarex by Bristol Myers Squibb ($2.4B), MedImmune by Astra Zeneca ($15.2B), and Wyeth by Pfizer ($68B).

Recombinant protein therapeutics are composed of 20 proteinogenic amino acids and metabolized by the same pathways used by endogenous proteins. Smaller proteins and peptides are cleared by renal excretion, larger proteins by phagocytic cells of the reticuloendothelial system. Proteins such as hormones, cytokines, or mAbs show exquisite specificity and sometimes bind their targets with picomolar affinity. To meet the need of large patient populations, therapeutic biologics are typically produced by genetically engineered bacteria or higher eukaryotic cells in large fermenters. Bacteria (e.g., E. coli) are used for production of nonglycosylated smaller proteins, such as insulin, whereas monoclonal antibodies (mAbs) are in most cases produced by eukaryotic cell clones (mostly based on Chinese hamster ovary cells) in order to obtain human-like glycosylation patterns and to achieve yields in the several grams/liters scale. Large-scale production of biologics is a highly sophisticated process requiring enormous investments in plants, documentation, and in-process controls.

Protein-based therapeutics can bind targets on the cell surface and in body fluids but are not able to reach intracellular targets in the cytoplasm or cell nucleus. This is, however, what small molecule drugs can achieve in addition to binding cell surface receptors and secreted proteins. Typically, small molecule drugs require a pocket or crevice to specifically and firmly bind their target and impact its functional activity. Thereby, they are uniquely suited to inhibit active centers of enzymes, like kinases, or to block or activate small ligand binding sites of nuclear or membrane-bound receptors. More challenging is the development of small molecule drugs that inhibit the interaction of two proteins. Small molecule drug development requires careful evaluation of physical properties and study of metabolism and pharmacokinetics (PK). These properties can hugely differ

Table 1. Protein-Based Therapeutics Enabled through Conjugation with PEG

Product	Annual Sales $M (2013)	Company
Adagen (Pegademase bovine)	25	Sigma-Tau
Oncaspar (mPEG-L-asparaginase)	27	Sigma-Tau
Krystexxa (pegloticase)	55	Savient
Pegasys (peginterferon alfa-2a)	1,400	Roche
PEG-inron (peginterferon alfa-2b	496	Merck
Cimzia (peg-anti-TNF-α antibody fragment)	790	UCB
Neulasta (pegfilgrastim)	4,400	Amgen
Mircera (methoxy polyethylene glycol-epoetin beta)	467	Roche
Somavert (pegvisomant)	58	Pfizer
Peginesatide (Omontys)	N/A	N/A

between closely related compounds and largely determine their tissue biodistribution, bioavailability, toxicity, metabolism, and half-life.

In this perspective, we review a particular aspect of drug development in which modification of a therapeutic protein with a chemical compound produces a new molecular entity in an effort to leverage the best properties of each class. Although it might seem counterintuitive that combining biologics and small molecule drugs into one molecule could be of benefit, given the fundamental differences between them, we describe examples where resulting small molecule/biologics conjugates exhibit superior properties. The examples that we will cover include proteins conjugated with biologically inert molecules, such as polyethylene glycol (PEG), to improve their PK properties; antibody drug conjugates (ADCs) and other proteins conjugated with biologically active small molecules and peptides; and radio immunoconjugates, (e.g., proteins conjugated with radio isotopes).

In order to develop these hybrid molecules, chemists and biologists need to learn how to best work together to solve the problems inherent in combining these two disparate systems. For example, most chemical reactions are designed with solvents and concentration that are not compatible with protein chemistry (Kalia and Raines, 2010, Stephanopoulis and Francis, 2011). On the other hand, protein chemistry utilizes immobilization and enzymatic catalysis that may not be compatible with desired regio- and chemoselective chemical reactions. Despite the fact that these hybrid therapeutics are still rare, we argue that this may be an area that could profit from more focused collaboration, exploration, and cross pollination between the fields.

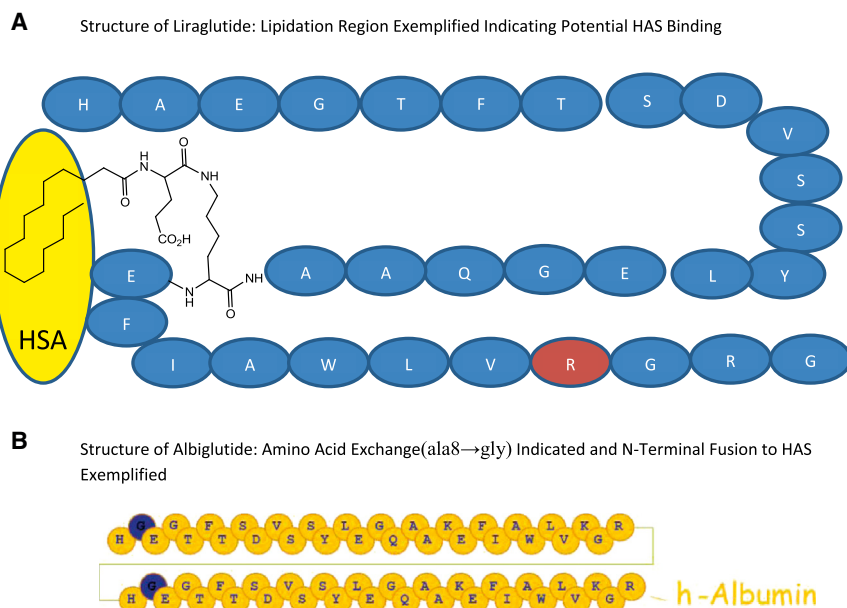## Tuning Proteins and Peptides by Chemical Conjugation of Polyethylene Glycol

A favorable property of therapeutic proteins—in particular of mAbs—is their long serum half-life. For instance, the human antibody denosumab (Prolia, Amgen) needs to be administered to women with postmenopausal osteoporosis only every 6 months (Adler and Gill, 2011). Most other mAb-based therapies are given at intervals of 1–4 weeks, which is extremely infrequent compared to daily dosing typical of oral small molecule drugs. Given their vulnerability to digestion in the gastrointestinal tract, protein-based therapeutics are injected subcutaneously (s.c.) or intravenously (i.v.). Subcutaneous administration often employs devices that improve convenience and may allow for self-administration by patients.

The long serum half-life of antibodies is mediated by their pinocytosis through endothelial cells followed by low-pH binding to the neonatal FcRn receptor within acidic endosomes, their recycling back to the cell surface, and ultimate release at neutral pH from FcRn (Zalevsky et al., 2010). One other protein that can use this highly efficient retention mechanism is human serum albumin (HSA), which in humans can circulate in blood for up to 3 weeks (Sleep et al., 2013). Most other serum proteins have half-lives in the range of hours to just a few days. If the protein molecular size is below the cut-off for renal clearance (approximately 50 kDa) serum half-life is only in the range of hours to minutes. This is the case for many therapeutic peptide hormones like interferons, insulin, granulocyte colony-stimulating factor (G-CSF), and interleukin-2 (IL-2), which require daily or multiple daily injections to ensure sufficient exposure. This situation was a main reason prompting the search for modifications that can increase serum half-life of small therapeutic proteins and peptides, ideally leaving the biological activity of the modified protein largely unencumbered. This should translate into less frequent dosing and more convenience for patients.

Polyethylene glycol, or PEG, is a biologically inert, water-soluble polymer with the general formula $C_{2n}H_{4n+2}O_{n+1}$. It is widely used in pharmaceutical and cosmetic products as well as in many other industries (Hawley, 2005). Given the well-explored properties of PEG, it was first selected in the 1970s by Frank Davis and colleagues for conjugation to proteins (Davis, 2002). The first PEGylated therapeutic protein, Adagen, was approved by the FDA in 1990. It is an adenosine deaminase inhibitor for treatment of severe combined immunodeficiency disease (Hershfield et al., 1987). Since then, several other PEGylated protein therapeutics followed with market approvals (Harris and Chess, 2003; Kang et al., 2009) (Table 1).

PEGylation of therapeutic proteins and peptides had numerous beneficial effects. By essentially wrapping the conjugated protein with a flexible, hydrophilic, and hydrated shell of PEG, the biophysical properties of proteins can be significantly altered. For instance, the modification can decrease access of proteolytic enzymes and of neutralizing antibodies to the protein core, thereby increasing stability and decreasing immunogenicity at the same time. The Stoke's radius of the protein is significantly increased by conjugated PEG, which reduces renal clearance and in turn prolongs serum half-life. Proteins that are hydrophobic or tend to aggregate become more soluble by PEGylation, further enhancing their stability and bioavailability. The most predictive and most desired property of conjugated PEG, however, is its impact on PK by increasing patient exposure or the area under the curve (AUC) of the protein in serum. From a commercial viewpoint, PEGylation can extend the life cycle and patent life of a therapeutic protein by creation of a novel drug product. In most cases, PEGylation was found to reduce the biological activity of the attached protein, presumably

A Structure of Liraglutide: Lipidation Region Exemplified Indicating Potential HAS Binding

B Structure of Albiglutide: Amino Acid Exchange(ala8→gly) Indicated and N-Terminal Fusion to HAS Exemplified

Figure 1. Half-Life Extension Technologies as Applied to GLP-1 Analogs for the Treatment of Type 2 Diabetes
(A) Structure of Liraglutide (Victoza): lipidation (palmitoylation) through a glutamic acid spacer on lysine 26, resulting in HSA binding in vivo and prolonged half-life (11–15 hr as opposed to 2 min for endogenous GLP-1 peptide).
(B) Structure of Albiglutide (Eperzan and Tanzeum): amino acid exchange (ala8→gly) to reduce deactivation due to dipeptidyl peptidase-4 and N-terminal fusion to HSA to extend half-life (4–7 days).

through the flexible polymer transiently getting in the way of desired protein-ligand interaction (Pack and Plückthun, 1992). However, increased AUC and higher dosing can compensate for a reduction in biological activity. Another potential issue of PEGylation is its metabolism. There seems to be no other pathway for elimination of the inert polymer than its limited renal clearance and storage in cellular vacuoles after the attached protein has been degraded (Rudmann et al., 2013).

The significance of PEGylation is best reflected by the number of PEGylated therapeutics on the market, impact on patients, annual sales potential, and the number of companies engaged in the technology. In 2013, 10 PEGylated drugs made between US $25M and $4.4B in annual revenues and, in aggregate, made more than $7.5B of revenues (Table 1). By these numbers, PEGylation clearly is the most significant and successful chemical modification of therapeutic proteins.

Beyond PEGylation
Recently, attempts have been made to replace PEG by recombinantly attaching hydrophilic amino acid sequences that have PEG-like properties (Schlapschy et al., 2013). These sequences have either a high content of proline or glycine in order to avoid formation of stable secondary structures. An obvious advantage of such recombinant fusions is that they are metabolized as regular proteins and do not require conjugation chemistry. Another approach that can be useful for extending serum half-life is the recombinant fusion of proteins to FcRn-binding Ig Fcγ domains. One very successful example is etanercept (Enbrel, Amgen), where two copies of the extracellular domain of the human TNFα receptor are fused to a human Fcγ1 domain. Enbrel is among the top ten best-selling therapeutics. Like PEGylation, Fcγ fusions can significantly change the biophysical properties of proteins. Fusion proteins must therefore be carefully designed and optimized for their pharmaceutical behavior.

Other technologies that enable half-life extension of therapeutic peptides or proteins are based on fusion to HSA, a natural, nonim-

munogenic plasma carrier protein, or to small molecules that bind to HSA (Subramanian et al., 2007). As described above, HSA uses the same FcRn-based retention mechanism as antibodies. Lysine, tyrosine, and the free thiol residues of HSA can be used for chemical conjugation to the drug product, with the free thiol at position 34 being most widely used. This approach is particularly useful for peptides where a maleimide linker has been synthetically introduced to specifically react with the free thiol, allowing for the formation of a stable thioether bond between albumin and peptide (Dennis et al., 2002). Alternatively, several small molecules bind with exceptionally high specificity to HSA and can be tethered to biological molecules to realize half-life extension, such as conjugation to the XTEN polymer (Podust et al., 2013). Lipidation or PASylation (Schlapschy et al., 2013). There are a few commercial applications of HSA binding that demonstrate the utility of these approaches: Liraglutide and Albiglutide (Figure 1). Liraglutide is produced by a process that includes expression of recombinant DNA in *S. cerevisiae* and has been engineered to be 97% homologous to native human GLP-1 by substituting arginine for lysine at position 34. Further chemical processing involves attaching a C-16 fatty acid (palmitic acid) with a glutamic acid spacer on the remaining lysine residue at position 26 of the peptide precursor. The lipid side chain thus introduced binds to albumin and provides extended half-life. Similar approaches that utilize the inherent lipophilic nature of the therapeutic molecule include abraxane, in which the lipophilic taxane small molecule is formulated with HSA. Albiglutide is a GLP-1 receptor agonist developed through the fusion of two repeats of human GLP-1 (7–36) molecules to recombinant human albumin. The GLP-1 dimer was used to avoid potential reductions of the interaction of the GLP-1 moiety of the monomer with its receptor in the presence of albumin. A single amino acid substitution (ala8→gly) renders the molecule resistant to dipeptidyl peptidase-4 (DPP-4). Albiglutide has a half-life of 4–7 days, much longer than other synthetic GLP-1 molecules, such as exenatide (Byetta) and liraglutide (Victoza). The utility of HSA binding as a mechanism for tuning pharmacokinetic properties of peptidic molecules has shown utility in the lab, clinic, and now in the practice of medicine and holds great promise for future applications.
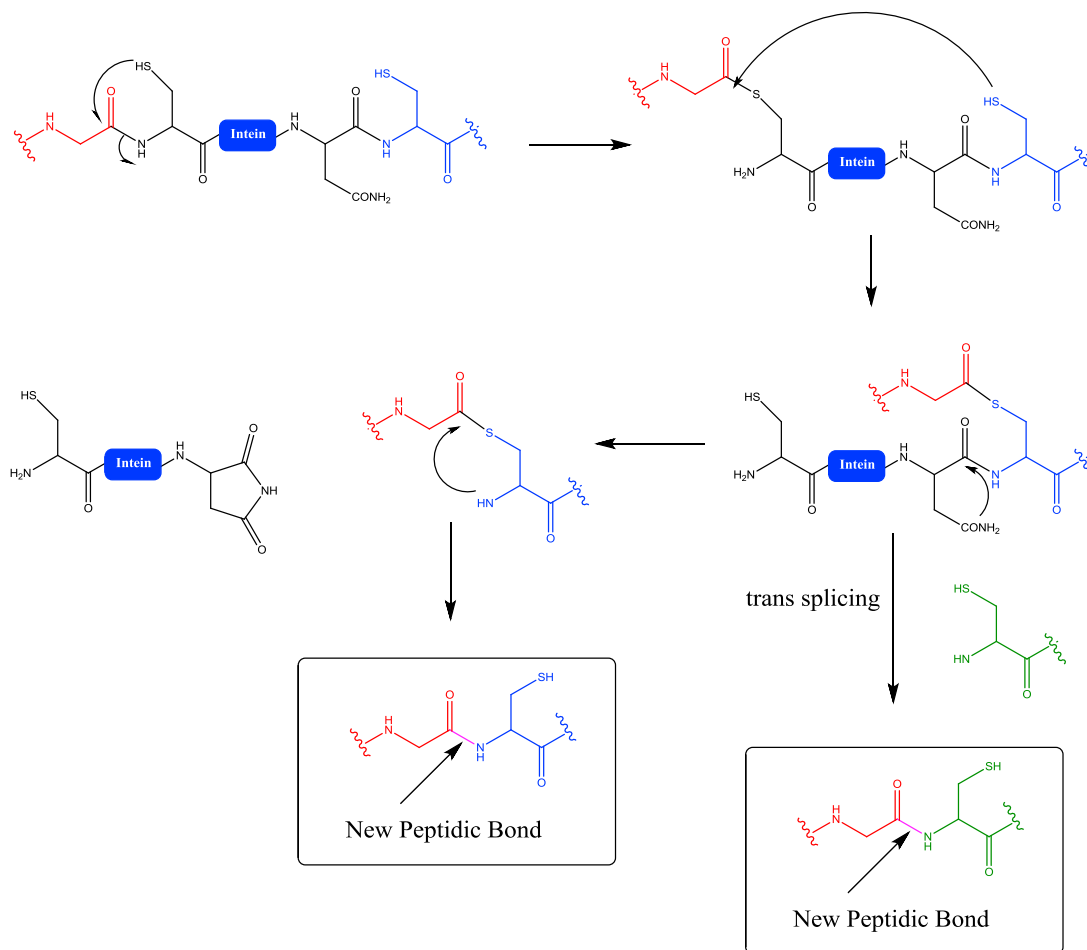
Future Technologies: Native Chemical Ligation and Expressed Protein Ligation
Native chemical ligation (Dawson et al., 1994) and expressed protein ligation (Shah and Muir, 2014) are two techniques that

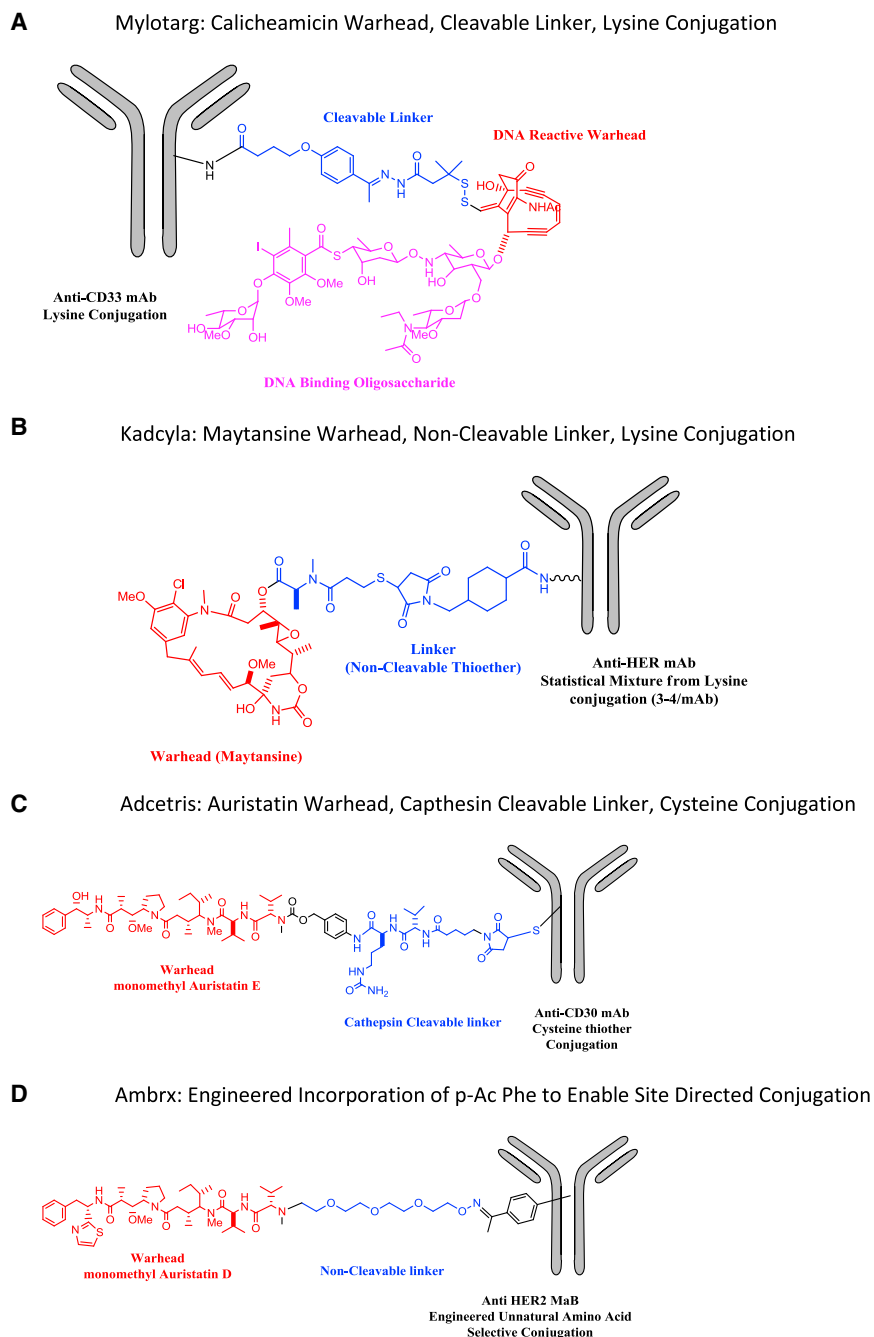**A** Native Chemical Ligation and Expressed Protein Ligation Utilizing Inteins

New Peptidic Bond

B Expressed Protein Ligation

trans splicing
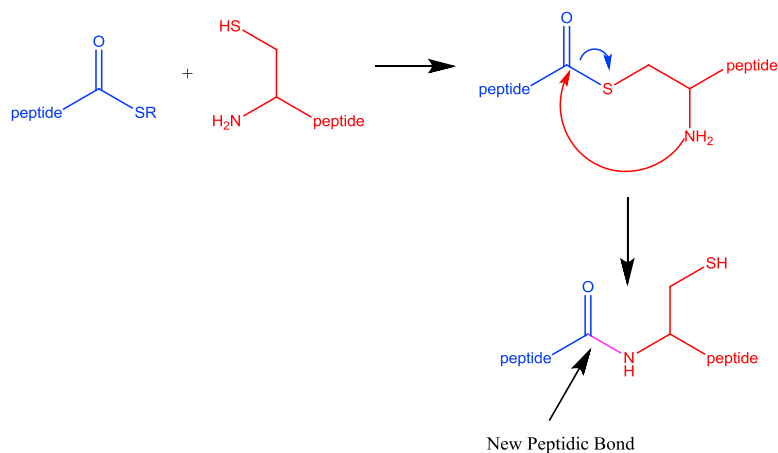
New Peptidic Bond

New Peptidic Bond

Figure 2. Mechanisms of Peptide and Protein Splicing
(A) Native chemical ligation that involves N-terminal cysteine trans-thioesterification followed by N-S rearrangement to form a new peptidic bond and (B) expressed protein ligation catalyzed by an intein subunit to facilitate S-N rearrangement followed by capture and succinimide formation. The capture of the thioester can be within the same protein or between an exogenously introduced N-terminal cysteine containing peptide, resulting in protein fusion products.

A   Mylotarg: Calicheamicin Warhead, Cleavable Linker, Lysine Conjugation

B   Kadcyla: Maytansine Warhead, Non-Cleavable Linker, Lysine Conjugation

C   Adcetris: Auristatin Warhead, Capthesin Cleavable Linker, Cysteine Conjugation

D   Ambrx: Engineered Incorporation of p-Ac Phe to Enable Site Directed Conjugation

Figure 3. Examples of Antibody Drug Conjugate Technology Indicating Warhead, Linker Type, and Method of Conjugation to mAb

(A) Mylotarg (gemtuzumab ozogamicin): calicheamicin warhead, cleavable (disulfide) linker, lysine conjugation (2–3 per IgG) to a mAb to CD33.

(B) Kadcyla (ado-trastuzumab emtansine): maytansine warhead (Dm-1), noncleavable linker (thioether), lysine conjugation (3–4 per igG) to amb that taregts HER2.

(C) Adcetris (Brentuximab vedotin: auristatin warhead (MMAE), capthesin cleavable linker, cysteine conjugation (3–5 per IgG) to a mAb that targets CD30.

(D) Ambrx: next-generation ADC technology platform whereby a HER2-specific mAb is site-specifically conjugated to a potent cytotoxic tubulin inhibitor. Site specificity is achieved through incorporation of p-Ac Phe to enable orthogonal chemical reactivity and site-directed conjugation.

tionally generates a thioester at the C terminus of the target protein. The intein fusion proteins are treated with peptides containing an N-terminal cysteine residue to effect native chemical ligation. The resulting product is a chemical conjugation of an expressed protein, although it is not evident by initial inspection because the "conjugate" is a peptide itself. The fusion is termed "traceless" because it incorporates protein fusion without any nonpeptidic reagents or linkers. The intermediate thio-ester can be trapped *in trans* by an exogenous synthetic peptide to create a new fusion protein. This very powerful tool has been utilized to make new biologically active fusion proteins and therapeutic agents. Future applications include utilizing these methods for "Biologics Structure Activity Relationship," which brings the insight of modern medicinal chemistry into the arena of biologics drug discovery (Fierz and Muir, 2012).

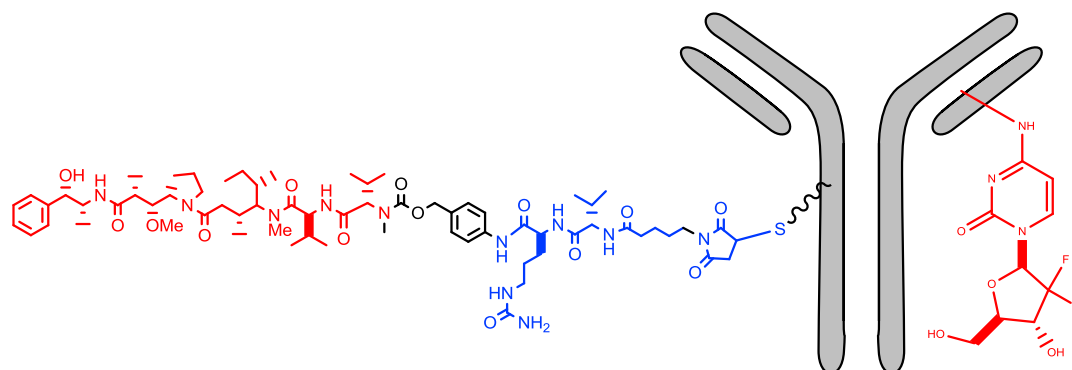## Chemical Conjugation of Toxins to Antibodies for Tumor Targeting

Chemotherapy of cancer often uses highly toxic, small molecule compounds with narrow therapeutic windows. One important class of chemotherapies targets tubulin, a component of the mitotic spindle. The issue with this class, as with a number of other available chemotherapeutic compounds, is their lack of specificity, as they affect both proliferating cancer and normal cells, leading to, among many other side effects, hair loss (alopecia), neurotoxicity, gastrointestinal bleeding, and/or immunodeficiency from bone marrow suppression. As mentioned above, mAbs can bind to cell surface antigens on cancer cells with high specificity and affinity. This prompted the idea to use monoclonal antibodies as a vehicle to precisely deliver highly toxic compounds to cancer

demonstrate a synergy between synthetic chemistry of peptides and protein expression and have recently been advanced by Kent, Muir, and colleagues (Shah and Muir, 2014). In native chemical ligation, an N-terminal cysteine residue reacts with a thioester to undergo transthioesterification followed by a rapid $S \rightarrow N$ acyl transfer to form a new amide bond (Figure 2). This reaction is a powerful tool for peptide ligation and hence protein synthesis.

Expressed protein ligation is an extension of native chemical ligation that couples the power of peptide synthesis with protein expression. In this method, a target protein is expressed as a fusion protein with an intein, a protein subunit that posttransla-

A Sutro Technology: Targeted Engineering and Multiple Warhead Conjugation

Drug1 on HC	Drug1 DAR	Drug2 on LC	Drug2 DAR	Total DAR
MMAF	1.8	SN-38	1.4	3.2
MMAF	1.8	PBD dimer	1.5	3.4
MMAF	1.9	Gemcitabie	1.6	3.6
PBD dimer	**1.9**	**MMAF**	**1.7**	**3.7**
Gemcitabine	2.0	MMAF	1.6	3.5

B Mersana Fleximer Technology: Multiple Toxins (>20) per Antibody Structure
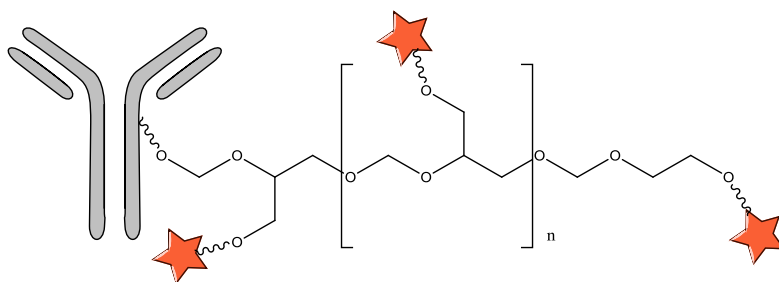
Figure 4. Emerging Technologies for Antibody Drug Conjugates
(A) Sutro Xpress CF technology can incorporate nonnatural amino acids (nnAA) in an antibody structure, thereby allowing for single-species ADCs with site-specific conjugation of linker and warhead. Additionally, multiple different warheads can be conjugated to a single antibody as is exemplified in the table.
(B) Mersana Fleximer technology allows multiple warheads (>20) to be conjugated on a polyal framework.

cells with the aim to increase therapeutic window and efficacy, while reducing dose levels and side effects (Chari et al., 2014). Quite a number of challenges had to be overcome on the 30 year path to the first commercial antibody drug conjugate (ADC) products. The efforts necessitated identification of ideal toxins to couple to antibodies; selection of the right antibodies targeting the most appropriate antigens; design of mAb/toxin linkers with ideal properties; optimization of chemical strategy to covalently attach small molecule toxins to the chosen antibody in a directed, efficient, stable and reproducible fashion; and controlling the release of the toxin from the ADC once it is delivered to the target cell.

While an enormous number of ADC programs are in preclinical and clinical development, only three ADCs have thus far reached clinical routine. One of them, for treatment of acute myelogenous leukemia in elderly patients, gentuzumab ozogamicin (Mylotarg, Pfizer; Sorokin, 2000), has been already retracted from the market because of a questionable risk/benefit ratio. The other two, brentuximab vedotin (Adcetris, Seattle Genetics) and ado-trastuzumab emtansine (Kadcyla or T-DM1, Roche), which were only recently approved by the FDA, showed high clinical activity in the treatment of Hodgkin's lymphoma (HL) and metastatic breast cancer, respectively. Adcetris' approval was based on a phase II clinical trial showing a response rate of >90% in refractory HL (Vaklavas and Forero-Torres, 2012). Kadcyla showed clinical responses and an increased overall survival in metastatic breast cancer patients who became refractory to the humanized anti-HER-2

Table 2. Companies Engaged in Developing ADC Technologies and Clinical Candidates

Company	Linker Technology	Warhead	Examples
Wyeth/Pfizer	disulfide cleavable linker	calicheamycin	gentuzumab ozogamicin, Inotuzumab ozogamicin (CMC-544; Mylotarg)
Immunogen	noncleavable maleimide thio Michael conjugation	maytansine	ado-trastuzumab emtansine (T-DM1 Kadcyla); lorvotuzumab mertansine (CD56, IMGN-901), SAR3419 (CD19)
Seattle Genetics	noncleavable maleimide thio Michael conjugation	auristatin (MMAE or MMAF)	Adcetris (CD30) CD22-specific CD79b-specific; GPNMB specific; PSMA specific linked to MMAE or MMAF
Sutro	cell-free expression of mAbs with nonnatural amino acids (nnAAs)	dual warhead conjugates	Celgene and Pfizer collaborations
Ambryx	EuCode expression system for nnAAs	multiple warheads (FK 506)	Astellas, BMS, Merck partnerships
Mersana	flexible linker technology	multiple	
Spirogen	pyrrolobenzodiazepine (PBD) technology	PDB	purchased by AZ in 2013
PolyTherics	ThioBridge technology	cytolysins	IPO launched in June 2014
CytomX	probody drug conjugates	maytansine	Pfizer collaboration

monoclonal IgG1 antibody trastuzumab, which constitutes the mAb backbone of the ADC (LoRusso et al., 2011).

Mylotarg, Adcetris, and Kadcyla use different strategies to chemically conjugate small molecule warheads to their respective antibody backbone. Mylotarg uses a cleavable linker to attach calicheamycin, a DNA double-strand breaking toxin, to an anti-CD33 monoclonal antibody. Adcetris uses a capthesin-cleavable linker to tether auristatin, a microtubule assembly inhibitor, to an anti-CD30 monoclonal antibody. Lastly, Kadcyla uses a noncleavable linker to attach maytansine, likewise a microtubule inhibitor, to the therapeutic anti-HER-2 antibody trastuzumab. Ambrx has taken the very bold approach of expanding the genetic code to allow the incorporation of the nonnatural amino acid p-acetyl-phenylalanine. This provides a ketone functionality within a peptidic network that has orthogonol reactivity and allows for highly selective and precise conjugation (Figure 3).

Preclinical models showed that small molecule payloads that were too toxic when administered on their own were tolerated and highly efficacious in tumor eradication when bound to a tumor-targeting monoclonal antibody (Junutula et al., 2008). Because only a minute fraction of administered ADCs will reach and bind tumor cells, the bulk of administered ADC needs to be tolerated and metabolized by normal tissues. This leads to dose-limiting off-target toxicities of ADCs including hepatotoxicity and bone marrow suppression. In addition, on-target toxicities are possible if normal tissues express the antibody's target antigen (McDonagh et al., 2008).

Based on two marketed products and multiple clinical programs, ADCs can be considered a valuable and successful class of drug products where biologics and chemicals are combined in a synergistic fashion. What will be decisive for the development of future and further improved ADCs is the choice of target antigens, choice of the most adequate linker chemistry, and a better defined and controlled attachment of toxins to the antibody (Jackson et al., 2014). Site-directed incorporation of unnatural amino acids has recently been shown to allow for an exquisitely directed conjugation of toxins to antibodies (Axup et al., 2012). The resulting ADCs were found to be more potent and differentiate themselves from molecules generated through random attachment to reactive natural amino acids (Tian et al., 2014; Flemming 2014). Others have focused on the covalent attachment of multiple different toxins to a single mAb, providing an orthogonal mechanism for cellular apoptosis. In addition, the company Mersana has recently demonstrated the feasibility of a multivalent tether that allows for significantly increasing the number of toxins attached per antibody (Papisov et al., 2005) (Figure 4). Currently, enormous efforts are ongoing in the industry to provide new ADC technologies and product candidates for cancer patients (Table 2).

Chelation of Radioisotopes to Antibodies for the Treatment of Cancer
Another variation of ADCs is radioimmunoconjugates (RICs). Here, the toxic payload is a radioactive isotope, like the beta emitters 131-iodine or 90-yttrium, that is typically attached to a chelating compound that is chemically conjugated to an antibody. This technology has also been used to produce diagnostic imaging reagents some time before the first RICs were developed for therapeutic use. Radioactively labeled antibodies allow the imaging of tumors expressing the respective target antigen of the antibody. The only two therapeutic RICs have been commercialized: Zevalin and Bexxar, both of which target the B cell antigen CD20 for the treatment of patients with non-Hodgkin lymphoma (NHL) (Figure 5).

Radiolabeled monoclonal antibodies have also been used with encouraging results in conjunction with stem cell transplantation in patients with hematologic malignancies targeting a variety of surface antigens including CD33, CD45, and CD66 for leukemias; CD20 and CD22 for non-Hodgkin's lymphomas; and ferritin for Hodgkin's disease (Pagel et al., 2002). Further work in this area has resulted in the emergence of "theragnostics," molecules that can be leveraged as diagnostics as well as therapeutics. In addition, utilizing these platforms as part of gold and
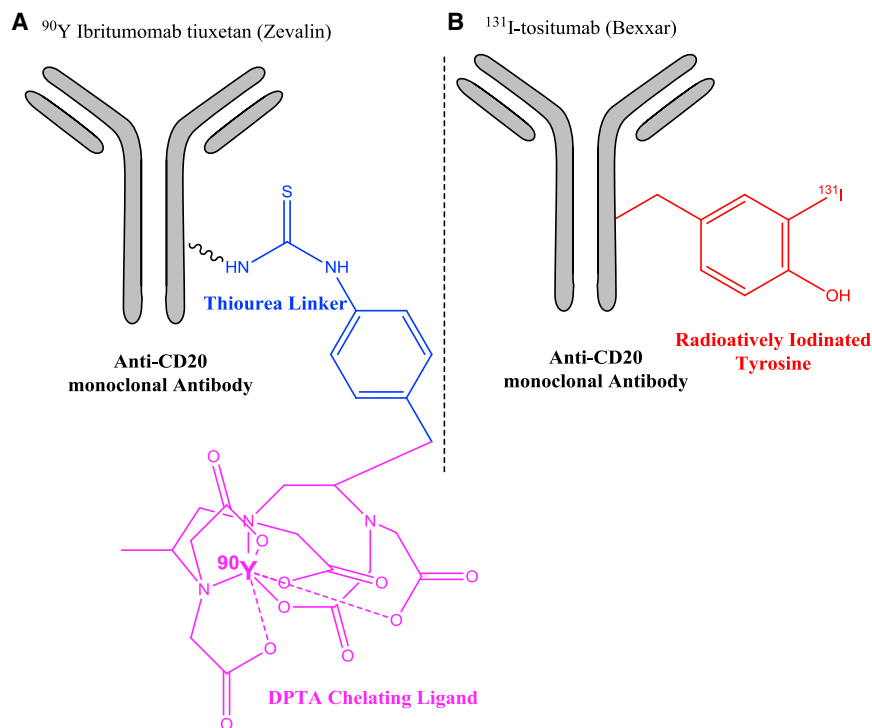
Figure 5. Representative Structure Radioimmunoconugates
(A) ^{90}Y Ibritumomab tiuxetan (Zevalin) thiourea linker DPTA chelating ligand for 90Y conjugated via lysine to a mAb targeted to CD20.
(B) ^{131}I-tositumab (Bexxar) iodinated tyrosine radioactive warhead in a mAb targeted to CD20.

iron nanoparticles has provided technologies that show promise for diagnosing and treating deadly cancers such as glioblastoma (Setua et al., 2014). Further exploration of this area should provide useful extensions of this technology.

Conclusions

Here we described a number of important human therapeutics that depends on the successful and synergistic combination of chemistry and biology. These combinations were greatly aided by the development of tailored coupling chemistries, an intelligent selection of both chemical entities and proteins and—as seems notorious in drug development—an enormous trial and error effort that lasted for decades. Just the few existing examples of successful combinations should inspire us to more systematicly look at our pharmaceutical and biotechnology industries from the perspective of combining the small molecule space with the protein universe. We think the opportunities that await in this area are substantial and that investing efforts to combine biologics and small molecules and solve their individual shortcomings will be fruitful and worthwhile from both a commercial and a clinical perspective.

REFERENCES

Adler, R.A., and Gill, R.S. (2011). Clinical utility of denosumab for treatment of bone loss in men and women. Clin. Interv. Aging 6, 119–124.

Axup, J.Y., Bajjuri, K.M., Ritland, M., Hutchins, B.M., Kim, C.H., Kazane, S.A., Halder, R., Forsyth, J.S., Santidrian, A.F., Stafin, K., et al. (2012). Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. Proc. Natl. Acad. Sci. USA 109, 16101–16106.

Chari, R.V.J., Miller, M.L., and Widdison, W.C. (2014). Antibody-drug Conjugates: an emerging concept in cancer therapy. Angew. Chem. Int. Ed. Engl. 53, 3796–3827.

Davis, F.F. (2002). The origin of pegnology. Adv. Drug Deliv. Rev. 54, 457–458.

Dawson, P.E., Muir, T.W., Clark-Lewis, I., and Kent, S.B. (1994). Synthesis of proteins by native chemical ligation. Science 266, 776–779.

Dennis, M.S., Zhang, M., Meng, Y.G., Kadkhodayan, M., Kirchhofer, D., Combs, D., and Damico, L.A. (2002). Albumin binding as a general strategy for improving the pharmacokinetics of proteins. J. Biol. Chem. 277, 35035–35043.

Fierz, B., and Muir, T.W. (2012). Chromatin as an expansive canvas for chemical biology. Nat Chem Biol. 8, 417–427.

Flemming, A. (2014). Antibody engineering: fine-tuning antibody-drug conjugates. Nat. Rev. Drug Discov. 13, 178.

Harris, J.M., and Chess, R.B. (2003). Effect of pegylation on pharmaceuticals. Nat. Rev. Drug Discov. 2, 214–221.

Hawley, G. (2005). Treasure in a Cornfield. (Kansas City: Paddle Wheel Publishing), pp. 175–186.

Hershfield, M.S., Buckley, R.H., Greenberg, M.L., Melton, A.L., Schiff, R., Hatem, C., Kurtzberg, J., Markert, M.L., Kobayashi, R.H., Kobayashi, A.L., et al. (1987). Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase. N. Engl. J. Med. 316, 589–596.

Jackson, D., Atkinson, J., Guevara, C.I., Zhang, C., Kery, V., Moon, S.J., Virata, C., Yang, P., Lowe, C., Pinkstaff, J., et al. (2014). In vitro and in vivo evaluation of cysteine and site specific conjugated herceptin antibody-drug conjugates. PLoS ONE 9, e83865.

Junutula, J.R., Raab, H., Clark, S., Bhakta, S., Leipold, D.D., Weir, S., Chen, Y., Simpson, M., Tsai, S.P., Dennis, M.S., et al. (2008). Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat. Biotechnol. 26, 925–932.

Kalia, J., and Raines, R.T. (2010). Advances in Bioconjugation. Curr. Org. Chem. *14*, 138–147.

Kang, J.S., Deluca, P.P., and Lee, K.C. (2009). Emerging PEGylated drugs. Expert Opin. Emerg. Drugs *14*, 363–380.

LoRusso, P.M., Weiss, D., Guardino, E., Girish, S., and Sliwkowski, M.X. (2011). Trastuzumab emtansine: a unique antibody-drug conjugate in development for human epidermal growth factor receptor 2-positive cancer. Clin. Cancer Res. *17*, 6437–6447.

McDonagh, C.F., Kim, K.M., Turcott, E., Brown, L.L., Westendorf, L., Feist, T., Sussman, D., Stone, I., Anderson, M., Miyamoto, J., et al. (2008). Engineered anti-CD70 antibody-drug conjugate with increased therapeutic index. Mol. Cancer Ther. *7*, 2913–2923.

Pack, P., and Plückthun, A. (1992). Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric FV fragments with high avidity in Escherichia coli. Biochemistry *31*, 1579–1584.

Pagel, J.M., Matthews, D.C., Appelbaum, F.R., Bernstein, I.D., and Press, O.W. (2002). The use of radioimmunoconjugates in stem cell transplantation. Bone Marrow Transplant. *29*, 807–816.

Papisov, M.I., Hiller, A., Yurkovetskiy, A., Yin, M., Barzana, M., Hillier, S., and Fischman, A.J. (2005). Semisynthetic hydrophilic polyals. Biomacromolecules *6*, 2659–2670.

Podust, V.N., Sim, B.C., Kothari, D., Henthorn, L., Gu, C., Wang, C.W., McLaughlin, B., and Schellenberger, V. (2013). Extension of *in vivo* half-life of biologically active peptides via chemical conjugation to XTEN protein polymer. Protein Eng. Des. Sel. *26*, 743–753.

Rudmann, D.G., Alston, J.T., Hanson, J.C., and Heidel, S. (2013). High molecular weight polyethylene glycol cellular distribution and PEG-associated cytoplasmic vacuolation is molecular weight dependent and does not require conjugation to proteins. Toxicol. Pathol. *41*, 970–983.

Schlapschy, M., Binder, U., Börger, C., Theobald, I., Wachinger, K., Kisling, S., Haller, D., and Skerra, A. (2013). PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. Protein Eng. Des. Sel. *26*, 489–501.

Setua, S., Ouberai, M., Piccirillo, S.G., Watts, C., and Welland, M. (2014). Cisplatin-tethered gold nanospheres for multimodal chemo-radiotherapy of glioblastoma. Nanoscale *6*, 10865–10873.

Shah, N.H., and Muir, T.W. (2014). Inteins: nature's gift to protein chemists. Chem Sci *5*, 446–461.

Sleep, D., Cameron, J., and Evans, L.R. (2013). Albumin as a versatile platform for drug half-life extension. Biochim. Biophys. Acta *1830*, 5526–5534.

Sorokin, P. (2000). Mylotarg approved for patients with CD33+ acute myeloid leukemia. Clin. J. Oncol. Nurs. *4*, 279–280.

Stephanopoulis, N., and Francis, M.B. (2011). Choosing an effective protein bioconjugation strategy. Nat. Chem. *7*, 876–884.

Subramanian, G.M., Fiscella, M., Lamousé-Smith, A., Zeuzem, S., and McHutchison, J.G. (2007). Albinterferon alpha-2b: a genetic fusion protein for the treatment of chronic hepatitis C. Nat. Biotechnol. *25*, 1411–1419.

Tian, F., Lu, Y., Manibusan, A., Sellers, A., Tran, H., Sun, Y., Phuong, T., Barnett, R., Hehli, B., Song, F., et al. (2014). A general approach to site-specific antibody drug conjugates. Proc. Natl. Acad. Sci. USA *111*, 1766–1771.

Vaklavas, C., and Forero-Torres, A. (2012). Safety and efficacy of brentuximab vedotin in patients with Hodgkin lymphoma or systemic anaplastic large cell lymphoma. Ther. Adv. Hematol. *3*, 209–225.

Zalevsky, J., Chamberlain, A.K., Horton, H.M., Karki, S., Leung, I.W., Sproule, T.J., Lazar, G.A., Roopenian, D.C., and Desjarlais, J.R. (2010). Enhanced antibody half-life improves *in vivo* activity. Nat. Biotechnol. *28*, 157–159.