mAbs 6:1, 34–45; January/February 2014; © 2014 Landes Bioscience

Site-specific antibody drug conjugates for cancer therapy

Siler Panowski, Sunil Bhakta, Helga Raab, Paul Polakis and Jagath R Junutula*

Genentech, Inc; South San Francisco, CA USA

Keywords: site-specific conjugation, antibody drug conjugate, THIOMAB, internalization, tumor antigen, linker, cytotoxic drug

Antibody therapeutics have revolutionized the treatment of cancer over the past two decades. Antibodies that specifically bind tumor surface antigens can be effective therapeutics; however, many unmodified antibodies lack therapeutic activity. These antibodies can instead be applied successfully as guided missiles to deliver potent cytotoxic drugs in the form of antibody drug conjugates (ADCs). The success of ADCs is dependent on four factors—target antigen, antibody, linker, and payload. The field has made great progress in these areas, marked by the recent approval by the US Food and Drug Administration of two ADCs, brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®). However, the therapeutic window for many ADCs that are currently in pre-clinical or clinical development remains narrow and further improvements may be required to enhance the therapeutic potential of these ADCs. Production of ADCs is an area where improvement is needed because current methods yield heterogeneous mixtures that may include 0–8 drug species per antibody molecule. Site-specific conjugation has been recently shown to eliminate heterogeneity, improve conjugate stability, and increase the therapeutic window. Here, we review and describe various site-specific conjugation strategies that are currently used for the production of ADCs, including use of engineered cysteine residues, unnatural amino acids, and enzymatic conjugation through glycotransferases and transglutaminases. In addition, we also summarize differences among these methods and highlight critical considerations when building next-generation ADC therapeutics.

Introduction

Monoclonal antibodies (mAbs) have long been an integral tool in basic research due to their high specificity and affinity for target antigens. For the past two decades, therapeutic mAbs have had substantial effects on medical care for a wide range of diseases, including inflammatory diseases and cancers. A critical feature of mAbs is their high specificity and their ability to bind target antigens, marking them for removal by methods such as complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC).[1] Antibodies can also impart therapeutic benefit by binding and inhibiting the function of target antigens, as in the case of trastuzumab (Herceptin®), bevacizumab (Avastin®), and cetuximab (Erbitux®).[2] However, antibodies against tumor-specific antigens often lack therapeutic activity.[3]

Conjugation to cytotoxic drugs or radionuclides can expand the utility of mAbs and improve their potency and effectiveness; the antibodies are thus used as a means to target and delivery a toxic payload to the selected diseased tissue. This approach is currently a major focus of therapeutic research. Antibodies have been conjugated to a number of cytotoxic drugs, though various linker chemistries and these antibody drug conjugates (ADCs) have the ability to selectively and potently kill antigen–expressing tumor cells in vitro and in xenograft studies.[4-6] ADCs have demonstrated success in the clinic, and there are now two such drugs, ado-trastuzumab emtansine (Kadcyla®) and brentuximab vedotin (Adcetris®), marketed in the United States. With over 30 ADCs currently undergoing clinical studies, it is likely that more conjugates will be approved in the future.

ADC development has been an iterative learning process, with ADCs evolving from murine antibodies that were conjugated to standard chemotherapeutic drugs to fully human antibodies conjugated to highly potent cytotoxic drugs. Our understanding of ADCs has improved substantially over the past 10 years and we now understand many of the critical factors required for their successful development, including target antigen selection, antibody, linker, and payload. One area of research that has seen recent advancement is that of conjugation chemistry. The implementation of site-specific conjugation, in which conjugation occurs only at engineered cysteine residues or unnatural amino acids for example, has resulted in homogeneous ADC production and improved ADC pharmacokinetic (PK) properties. This review will focus on current methods of site-specific conjugation, as well as the history and our present understanding of ADCs.

Antibody-Drug Conjugates

The history of ADCs

Historically, the use of drugs for the treatment of cancer has centered on chemotherapies that target rapidly dividing cancer cells. These chemotherapy drugs included the folate and purine analogs (methotrexate, 6-mercaptopurine), microtubule polymerization inhibitors/promoters (vinca alkaloids, taxanes) and DNA damaging agents (anthracyclines, nitrogen mustard).[7] These compounds target cancer cells but also other dividing cells in the body, and patients receiving treatment experience severe side

*Correspondence to: Jagath R Junutula; Email: jagath@gene.com
Submitted: 09/20/2013; Revised: 10/30/2013; Accepted: 10/31/2013
http://dx.doi.org/10.4161/mabs.27022

effects that greatly limit the administrable dose. The therapeutic index (maximum tolerated dose/minimum efficacious dose) for these drugs is small, resulting in a narrow therapeutic window (**Fig. 1**). To circumvent this obstacle in drug development and improve therapeutic index, researchers turned to ADCs. The promise of ADCs was that they could selectively deliver toxic compounds to diseased tissue, a concept first described by Paul Ehrlich as "Magic Bullets" in the early 1900s.[8]

ADC development, however, was not straightforward and those studied in the 1980s and early 1990s faced a number of challenges. Several early attempts at ADC development included the KS1/4 antibody-methotrexate conjugate for non-small cell lung cancer and the BR96 antibody-doxorubicin conjugate for metastatic breast cancer.[9,10] Both drugs were evaluated in the clinic, but despite localizing to tumors, the conjugates showed little or no therapeutic benefit.[11,12] Poor target antigen selection was likely a primary reason for the failure of these early conjugates. The antigens targeted by KS1 and BR96 were initially selected because their expression was associated with cancer cells, but both antigens were also expressed in normal tissues, resulting in toxicity.[11,13] Other factors that limited the success of these conjugates were the use of either chimeric or murine antibodies, which can elicit an immunogenic response, and the use of lower potency drugs.

Wyeth and Celltech improved on these early ADCs with the development of gemtuzumab ozogamicin (Mylotarg®), an anti-CD33 conjugate for the treatment of acute myeloid leukemia (AML). Gemtuzumab ozogamicin incorporated a highly potent calicheamicin derivative to help improve efficacy and a humanized antibody to limit immunogenicity,[14] but the mAb-drug linker was unstable and released 50% of bound drug in 48 h. Although gemtuzumab ozogamicin demonstrated promising activity in the clinic and was granted accelerated approved by the US Food and Drug Administration (FDA) in 2000, the drug was later withdrawn from the market after subsequent clinical data raised concerns about safety and clinical benefit when combined with the frontline standard of care.[15,16]

Lessons learned from the initial ADC programs mentioned above were incorporated into the development and design of second-generation ADCs, and two of these, brentuximab vedotin and ado-trastuzumab emtansine, showed impressive clinical efficacy and safety, and were recently approved by the FDA. Brentuximab vedotin, developed by Seattle Genetics in partnership with Millennium/Takeda for the treatment of anaplastic large cell lymphoma and Hodgkin lymphoma, chemically couples an anti-CD30 chimeric antibody with the highly potent antimitotic agent, monomethyl auristatin E (MMAE) through a protease cleavable linker.[17] Ado-trastuzumab emtansine, developed by Genentech with ImmunoGen's ADC linker-drug technology, targets human epidermal growth factor receptor 2 (Her2)-positive breast cancer and combines an anti-Her2 antibody (trastuzumab) with the cytotoxic agent maytansine (DM1) via a stable linker.[18] Knowledge gained from the development of these and other ADCs has led to a better understanding of the ways in which ADCs function and their clinical performance.

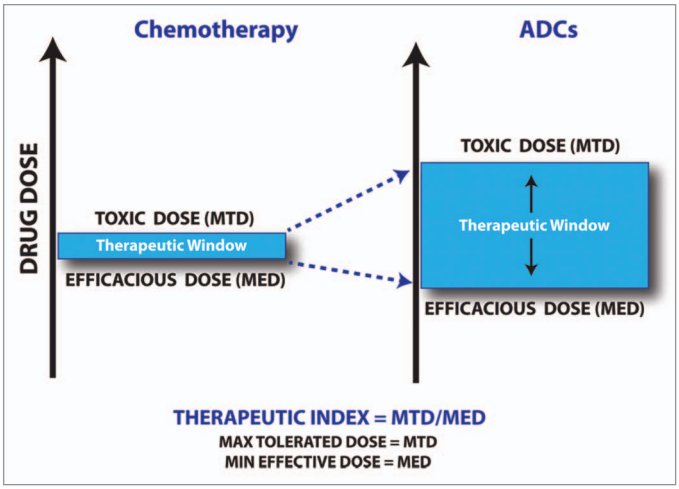

Figure 1. ADCs expand the therapeutic window. ADC therapeutics can increase efficacy and decrease toxicity in comparison to traditional chemotherapeutic cancer treatments. Select delivery of drugs to cancer cells increases the percent of dosed drug reaching the tumor, thus lowering the minimum effective dose (MED). The maximum tolerated dose (MTD) is increased, as less drug reaches normal, non-target tissue due to targeted delivery by the antibody. Taken together, the therapeutic window is improved by the use of ADCs.

ADC Function and Mechanism of Action

ADCs are designed to kill cancer cells in a target-dependent manner and the first step in this process is binding of the antibody to its antigen. The tumor antigen must be localized to the cell-surface so it can be accessed by a circulating antibody. Upon ADC binding, the entire antigen-ADC complex is internalized through receptor-mediated endocytosis (**Fig. 2**). This process generally occurs when a ligand binds a cell-surface receptor and initiates a cascade of events, including recruitment of adaptins and clathrin, inward budding of the plasma membrane, formation of early endosomes, and lastly trafficking to late endosomes and lysosomes.[19] Once inside lysosomes, ADCs are degraded and free cytotoxic drug is released into the cell, resulting in cell death. The mechanism of action of cell death can vary based on the class of cytotoxic drug used (e.g., disruption of cytokinesis by tubulin polymerization inhibitors such as maytansines and auristatins, DNA damage by DNA interacting agents such as calcheamicins and duocarmycins).[20] Neighboring cancer cells may also be killed when free drug is released into the tumor environment by the dying cell in a process known as the bystander effect.[21] For ADCs to work, a threshold level of free toxic drug must be reached inside and around tumor cells. Factors that influence whether this threshold is met, and thus determine the success of an ADC, include the target tumor antigen, antibody, linker and cytotoxic drug (**Fig. 3**).

Anatomy of ADCs

Importance of the tumor antigen

As mentioned earlier, the ideal tumor antigen must be localized to the cell-surface to allow ADC binding. Preferably the antigen also displays differential expression between tumor and normal

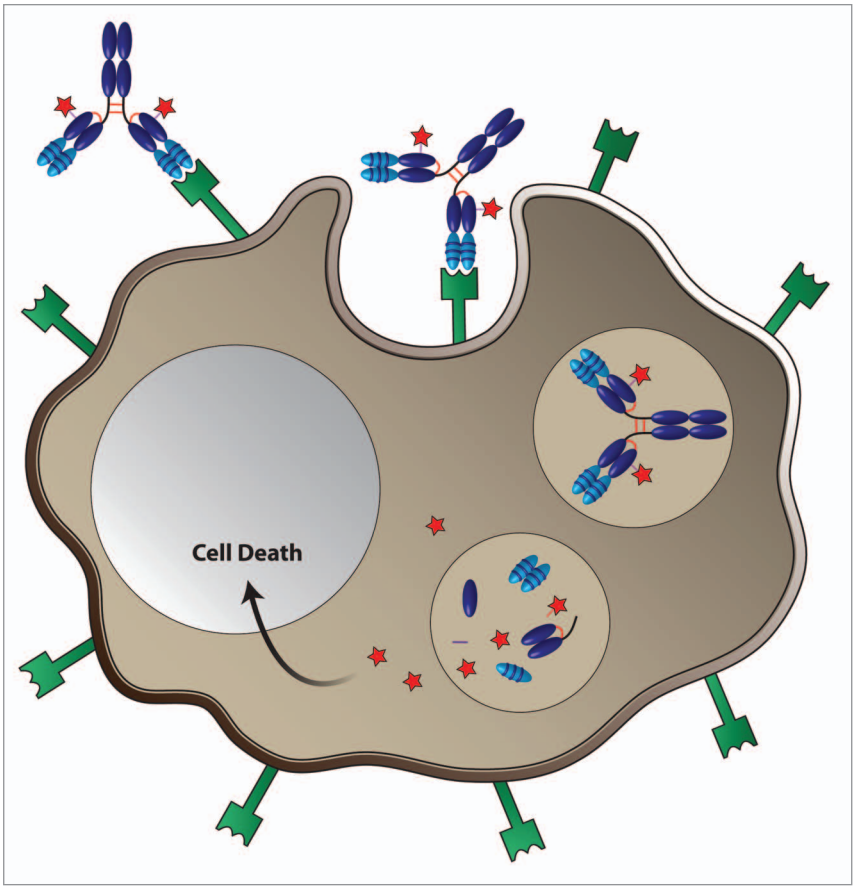

Figure 2. Delivery of cytotoxic drugs to cancer cells by ADCs. The monoclonal antibody component of an ADC selectively binds a cell-surface tumor antigen, resulting in internalization of the ADC-antigen complex through the process of receptor-mediated endocytosis. The ADC-antigen complex then traffics to lysosomal compartments and is degraded, releasing active cytotoxic drug inside the cell. Free drug causes cell death through either tubulin polymerization inhibition or DNA binding/damage depending on the drugs mechanism of action.

tissue, with increased expression in cancer cells. Expression of an antigen in normal tissue could enhance uptake of conjugate by the tissue, resulting in toxicity and lowering the dose of conjugate available to the tumor. Another important characteristic of the tumor antigen is ability to internalize upon ADC binding. The internalization of an ADC-antigen complex through receptor-mediated endocytosis, followed by ADC degradation in the lysosome, results in optimal free drug release and effective cell killing. That endocytosis will occur is not guaranteed for all cell-surface antigens, and the rate of internalization can vary from rapid to zero. Minimal ADC recycling to the cell surface and enhanced delivery of an internalized antigen/ADC to the lysosome also needs to occur for the maximal release of toxic free drug into the cell. Therefore, the ideal tumor antigen should be cell-surface expressed, highly upregulated in cancer tissue, internalized upon ADC binding, and able to release the cytotoxic agent inside the cell.[22]

Antibody specificity, affinity, and pharmacokinetics

Another critical factor that influences ADC success is the antibody itself. Even the perfect tumor antigen cannot be targeted if the antibody selected does not contain several crucial attributes. High specificity of the antibody for the tumor antigen is essential. An antibody that cross-reacts to other antigens or displays general non-specific binding can be taken up in normal tissues unpredictably and in high amounts, resulting in both toxicity and removal/elimination of the ADC before it can reach the tumor.[5,11,13] The antibody must also bind the target antigen with high affinity (K_d < 10 nM) for efficient uptake into target cells and it should be minimally immunogenic. An immune response mounted against an ADC, such as human anti-mouse antibodies (HAMA) against a murine ADC, can prevent repeat cycles of therapy.[23] It is also important to select an antibody with optimal PK properties (longer half-life with slower clearance in plasma).[24] Lastly, it should be noted that unknown factors related to the antibody appear to contribute to ADC activity, as demonstrated in a study where only two of seven antibody conjugates that bind CD22 were effective in vivo, a dramatic result not likely due to PK properties alone.[25]

Linker selection and intracellular drug release

The next step after tumor antigen identification and antibody development is selection of a suitable linker/cytotoxic drug. As might be expected, the drug plays a major role in ADC activity and characteristics. What might be less intuitive is that the linker between the antibody and drug also is very important. An ideal linker should be stable in circulating blood, but allow rapid release of active free drug inside tumor cells. If a linker is not stable in blood, drug will be lost and ADC activity will be decreased.[15,26]

Current linker formats that are being evaluated can be broadly categorized into two groups: cleavable linkers (acid-labile linkers, protease cleavable linkers, and disulfide linkers) and non-cleavable linkers. Acid-labile linkers are designed to be stable at pH levels encountered in the blood, but become unstable and degrade when the low pH environment in lysosomes is encountered (e.g., gemtuzumab ozogamicin). Protease-cleavable linkers are also designed to be stable in blood/plasma, but rapidly release free drug inside lysosomes in cancer cells upon cleavage by lysosomal enzymes. They take advantage of the high levels of protease activity inside lysosomes and include a peptide sequence that is recognized and cleaved by these proteases, as occurs with a dipeptide Val-Cit linkage that is rapidly hydrolyzed by cathepsins (e.g., brentuximab vedotin).

A third type of linker under consideration contains a disulfide linkage. This linker exploits the high level of intracellular reduced glutathione to release free drug inside the cell (e.g., the anti-CD56-maytansine conjugate IMGN-901). Linkers in

the non-cleavable category provide high stability in the blood, but are solely dependent on internalization, lysosomal delivery, and degradation of the ADC complex to release active drug and kill cancer cells (e.g., ado-trastuzumab emtansine). They may not release drug in extracellular space and are incapable of killing neighboring tumor cells through the by-stander effect.[27] Furthermore, optimal linker selection depends on the target antigen that is chosen. It was demonstrated that ADCs with cleavable linkers against seven B cell targets (CD19, CD20, CD21, CD22, CD79b, and CD180) showed in vivo efficacy. In contrast, only target antigens that were internalized and efficiently trafficked to lysosomes (CD22 and CD79b) displayed in vivo efficacy with non-cleavable linkers.[28] The specificity of free drug release in cells is a main goal of all of the linkers, and it is important for controlling the toxicity of the highly potent drugs used to construct ADCs. However, the balancing act between efficacy and toxicity varies for the above-mentioned linkers and linker selection will ultimately depend on experimentally determining the optimal combination of the correct linker, the target antigen and desired payload.

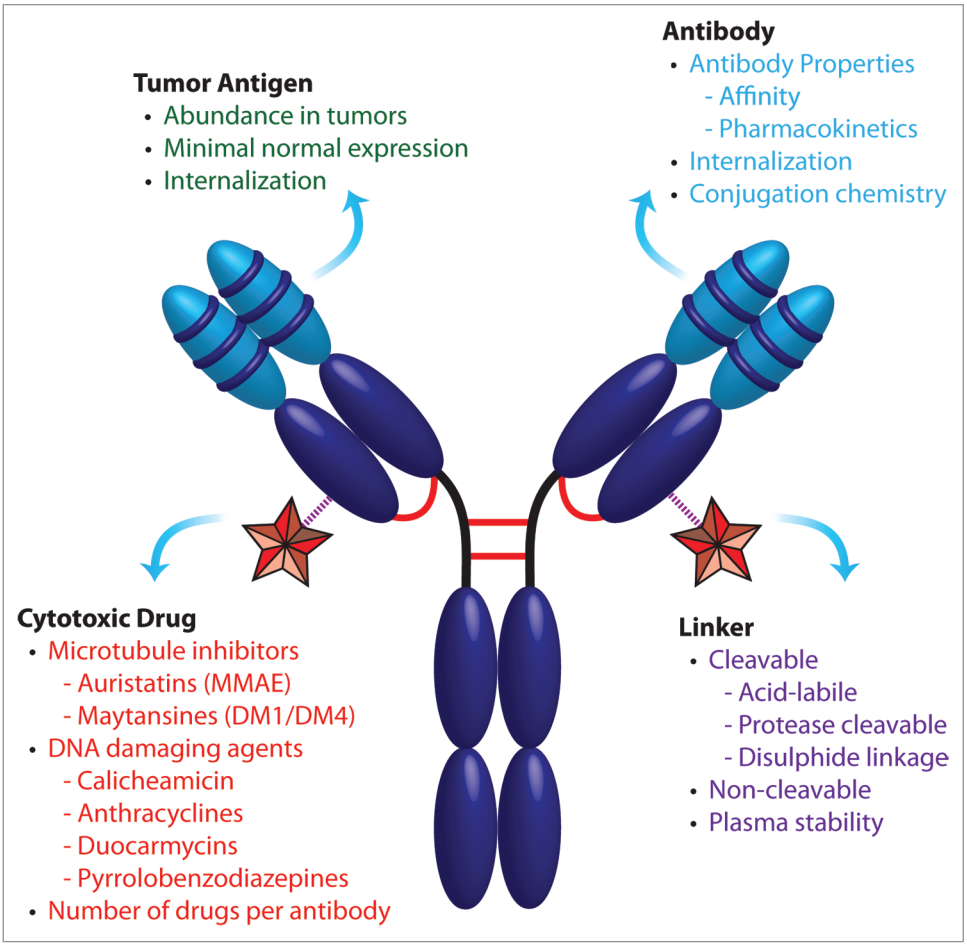

Figure 3. Critical factors that influence ADC therapeutics. ADCs consist of a cytotoxic drug conjugated to a monoclonal antibody by means of a select linker. These components all affect ADC performance and their optimization is essential for development of successful conjugates.

Cytotoxic drugs

The success of an ADC also depends on the use of an optimal drug. The percent of an injected antibody that localizes to a solid tumor is very small (0.003–0.08% injected dose per gram of tumor); therefore, toxic compounds with sub-nanomolar potency are desirable.[29] In addition, drugs must contain a suitable functional group for conjugation and need to be stable under physiological conditions. The drugs currently being used to construct ADCs generally fall into two categories: microtubule inhibitors and DNA-damaging agents. It should be noted that other drugs such as the polymerase II inhibitor, α-amanitin, are also under investigation.[30]

Microtubule inhibitors bind tubulin, destabilize microtubules, and cause G2/M phase cell cycle arrest. Auristatins and maytansinoids are two classes of microtubule inhibitors currently used in ADC development. MMAE is a highly potent auristatin (free drug IC_{50}: 10^{-11}-10^{-9} M) developed by Seattle Genetics and used in brentuximab vedotin, and DM1 is a highly potent maytansinoid (free drug IC_{50}: 10^{-11}–10^{-9} M) developed by ImmunoGen and used in ado-trastuzumab emtansine.[23,31-34]

DNA-damaging agents include anthracyclines, calicheamicins, duocarmycins, and pyrrolobenzodiazepines (PBDs). All of these drugs function by binding the minor groove of DNA and causing DNA stand scission, alkylation, or cross-linking. The cytotoxins are highly potent, with free drug IC_{50} of $<10^{-9}$ M, and ADCs that incorporate these agents have been explored in the clinic, including inotuzumab ozogamicin, an anti-CD22-calicheamicin conjugate developed by Pfizer, and MDX-1203, an anti-CD70-duocarmycin developed by Bristol-Myers Squib.[14,20,35-38]

The evolution of ADCs from BR96-doxorubicin and KS1/4-methotrexate to the currently marketed brentuximab vedotin and ado-trastuzumab emtansine exemplifies the substantial efforts and innovation of many scientists in the ADC field, and required optimization of all components of ADCs, including antibodies, linkers, and payloads. Successful ADC development depends on optimization of the delicate balance between efficacy and toxicity (target dependent and independent). (**Fig. 4**). However, the work is far from over, and further development may be essential to the success of many future ADC products. One area of current research that will help us take the next step in ADC evolution is site-specific conjugation.

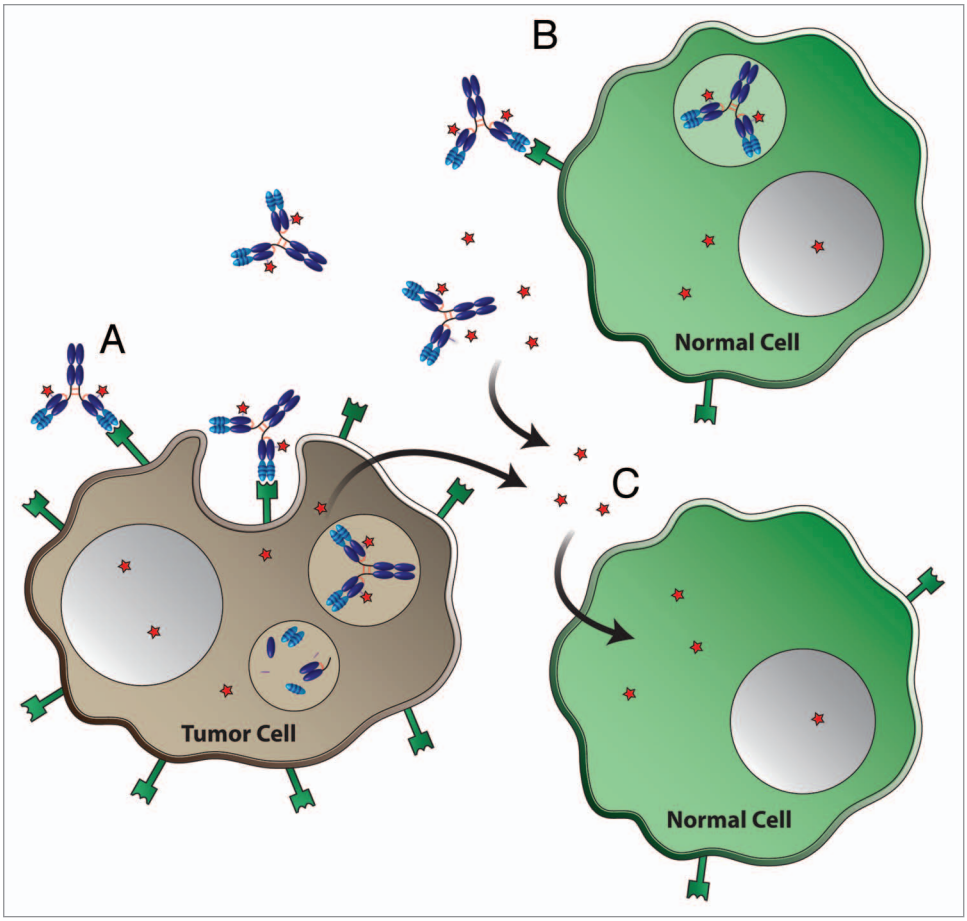

Figure 4. ADC metabolism in vivo. The therapeutic window of an ADC depends on the optimization of the delicate balance between efficacy and toxicity. The desired effect of ADCs is the target-dependent killing of tumor cells expressing high levels of target antigen (**A**). Side effects can be caused by target-dependent toxicity and killing of normal cells expressing low levels of target antigen (**B**), or by target-independent toxicity caused by entry of free drug into normal cells (**C**). Free drug can be released by ADC catabolism or by unstable labile linkers in the plasma..

Site-Specific Conjugation

Conventional ADC conjugation processes

Traditionally, conjugation of linker-drugs to an antibody takes place at solvent accessible reactive amino acids such as lysines or cysteines derived from the reduction of inter-chain disulfide bonds in the antibody. Lysine conjugation results in 0–8 conjugated molecules per antibody (**Fig. 5**), and peptide mapping has determined that conjugation occurs on both the heavy and light chain at ~20 different lysine residues (40 lysines per mAb). Therefore, greater than one million different ADC species can be generated.[6,39-41] Cysteine conjugation occurs after reduction of four inter-chain disulfide bonds, and the conjugation is thus limited to the eight exposed sulfhydryl groups. Linker-drugs per antibody can range from 0–8 (**Fig. 5**), generating more than one hundred different ADC species.[42] The diversity in heterogeneity of an ADC mixture is 2-fold because these ADC species differ in drug load and conjugation site. Therefore, each species may have distinct properties, which may result in a wide range of in vivo PK properties. In addition, batch-to-batch consistency in ADC production can be challenging and may require diligent manufacturing capabilities. Site-specific conjugation, in which a known number of linker-drugs are consistently conjugated to defined sites, is one way to overcome these challenges.[43,44] Heterogeneity is minimized and ADC properties are more predictable, with consistent conjugate production from batch to batch. Drug-to-antibody ratio (DAR) is precisely controlled and can be tailored to various linker-drugs, producing either 2- or 4-DAR site-specific ADCs (**Table 1**). Thus, site-specific conjugation is a major improvement to ADC drug development and it is no surprise that researchers have focused on a number of methods to achieve site-specific conjugation.

Site-specific conjugation through engineered cysteine residues

The amino acid cysteine contains a reactive thiol group that serves essential roles in the structure and function of many proteins. Conjugation of thio-reactive probes to proteins through cysteine residues has long been a method for protein labeling, and it has also been applied to the generation of ADCs. As described above, this process involves partial reduction of inter-chain disulfide bonds and results in a heterogeneous mixture of ADCs that differ with respect to site of conjugation, number of drugs per antibody, and number of intact inter-chain disulfide bonds.[42]

To avoid the problem of heterogeneity and to maintain disulfide bonds, cysteine residues can be engineered into proteins, but there are still many challenges to this approach. Engineered free cysteine residues on the surface of a protein can pair with cysteines on other molecules to form protein dimers.[45] It is also possible that introduced cysteines can pair intra-molecularly with native cysteine residues to create improper disulfide bonds, resulting in disulfide bond shuffling and possibly protein inactivation.[46] The success of using introduced cysteine residues for site-specific conjugation relies on the ability to select proper sites in which cysteine-substitution does not alter protein structure or function. To accomplish this, the Phage Elisa for Selection of Reactive Thiols (PHESELECTOR) was developed by introducing reactive cysteine residues into an antibody-Fab (trastuzumab-Fab 4D5) at various sites, displaying the Fab on phage, and screening to identify reactive cysteines that do not interfere with antigen binding.[47]

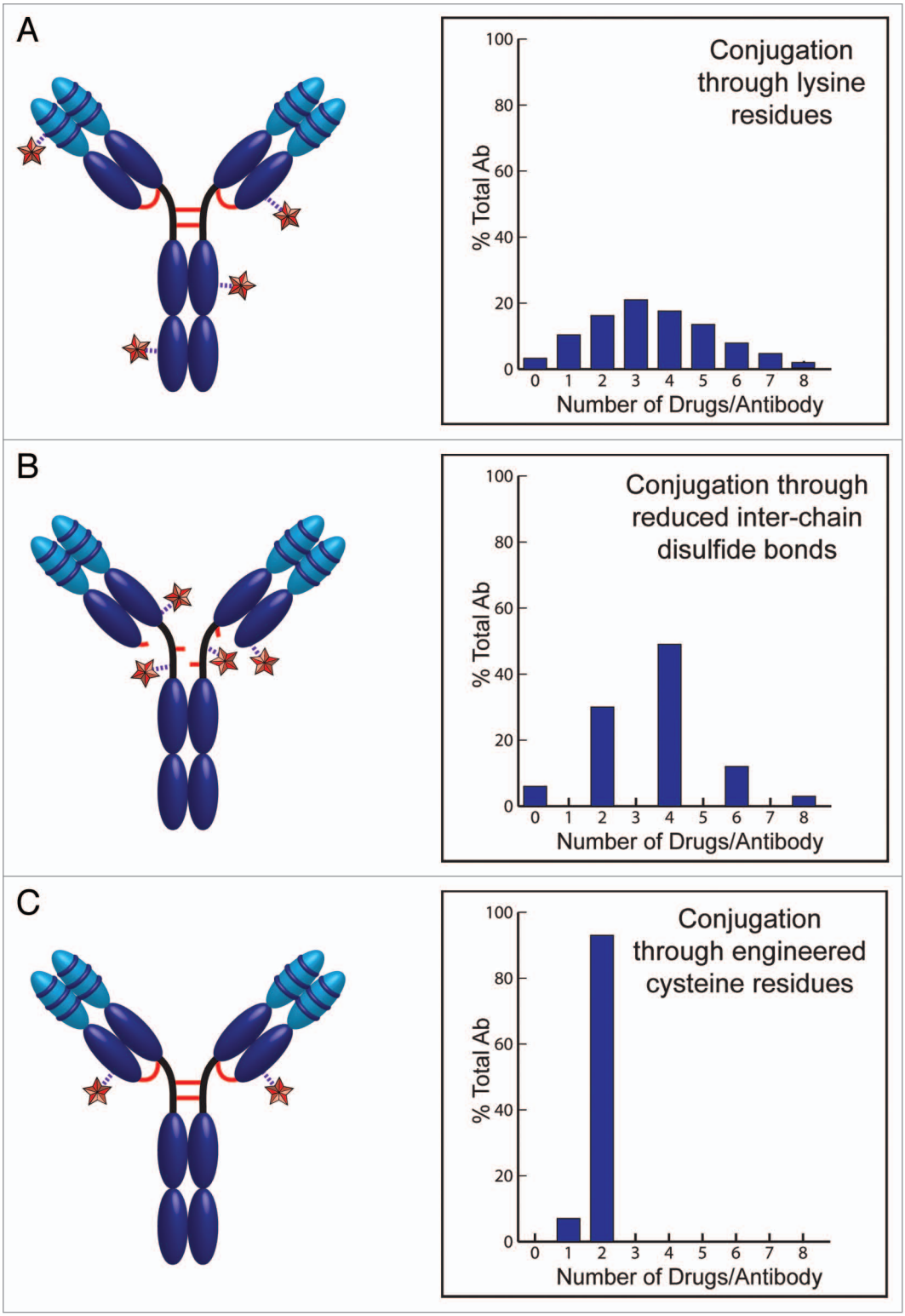

Figure 5. Conjugation methods for ADC development. ADC production using traditional conjugation through lysine residues or reduction of inter-chain disulfide bonds results in high heterogeneity in both drug to antibody ratio (DAR) and location of conjugation site. Site-specific conjugation greatly decreased this heterogeneity. (**A**) Lysine conjugation results in a DAR of 0–8 and potential conjugation at ~40 lysine residues/mAb. (**B**) Conjugation through reduced inter-chain disulfide bonds results in a DAR of 0–8 and potential conjugation at eight cysteine residues per mAb. (**C**) Site-specific conjugation utilizing two engineered cysteine residues results in a DAR of 0–2 and conjugation at two sites/mAb. DAR can be doubled by engineering four sites if desired. Data displayed in graphs were re-plotted from previous publications.[40,41,43]

Table 1. Methods used for generation of site-specific ADCs

	Engineered Cysteine Residues	Unnatural Amino Acids	Selenocysteine	Enzymatic Conjugation (Glutamine Tag, Glycoengineering, FGE)
Antibody engineering required	Cysteine substitution	Amber stop codon substitution	Addition of Sec insertion sequence	Addition of glutamine tag or aldehyde tag, none for glycoengineering or for pre-existing glutamine tag (Gln-295)
Cell line engineering required	None	Cell line expressing orthogonal tRNA/aaRS	None	Cell line overexpressing Formylglycine Generating Enzyme (FGE) for FGE method, none for other methods
Additional reagents required at time of antibody expression	None	Unnatural amino acids	Sodium Selenite	None
Enzymes required for conjugation	None	None	None	Glycotransferase, Transglutaminase,
Conjugation site location	Any location	Any location	C-terminus (other locations unknown)	Asn-297 for glycoengineering, Pre-existing glutamine tag (Gln-295) or any location for other methods
Drug-to antibody ratio (DAR)	2 or 4	2 or 4	2	2 for glycoengineering, 2 or 4 for glutamine tag and FGE
Conjugation chemistry	Maleimide, Bromoacetamide	Oxime, Click chemistry	Maleimide	Click chemistry, transamidation, hydrozino-Pictet-Spengler chemistry
Institutions exploring site-specific antibody conjugation methods	Genentech, MedImmune, Seattle Genetics	Allozyne, Ambrx, Sutro	National Cancer Institute	Innate Pharma, Glycos, Pfizer, Redwood Bioscience, SynAffix

To determine the generality and validity of this approach with full-length mAbs, conjugation of a cytotoxic drug to an anti-MUC16 mAb was investigated.[43] Based on PHESELECTOR assay results, heavy chain alanine 114 (Kabat numbering) was selected as an optimal site for cysteine substitution. Unlike conventional cysteine conjugation, in which drug was conjugated to both the heavy and light chain of the antibody, conjugation using the engineered cysteine site occurred only on the heavy chain at engineered cysteine residues, with greater than 92% of the engineered thio antibody (THIOMAB) conjugates containing two drugs (**Fig. 5**). Based on these results, conjugation to the engineered cysteine was both efficient and specific, especially compared with conventional cysteine conjugation. Importantly, substitution of cysteine at this position did not alter antigen binding of the HC-A114C anti-MUC16 THIOMAB compared with the original anti-MUC16 antibody. These results are significant because they demonstrate that the optimal sites for cysteine conjugation found using an anti-HER2 Fab and the PHESELECTOR method can also be applied to full-length antibodies, and data now suggest that these sites work well for site-specific conjugation to other mAbs (trastuzumab THIOMAB, anti-CD22 THIOMAB, anti-Steap1 THIOMAB and anti-TenB2 THIOMAB).[48-50]

The importance of site-specific conjugation was next highlighted by comparing the therapeutic windows of traditional anti-MUC16 drug conjugates (ADCs) and HC-A114C engineered anti-MUC16 THIOMAB drug conjugates (TDCs). Efficacies of the two conjugates were compared and despite having a decreased drug load (~2 drugs per TDC vs ~3.5 drugs per ADC), the site-specific THIOMAB conjugates were as active and efficacious in both in vitro and in vivo studies, thus providing equivalent efficacy at half the drug dose. Interestingly, engineered site-specific TDC conjugates were also better tolerated in both rat and cynomolgus monkey toxicity models compared with traditional

ADC conjugates.[40,43] Animals administered anti-MUC16 site-specific TDCs displayed reduced liver and bone marrow toxicity compared with conventional ADCs. Taken together, the above results demonstrate that site-specific TDCs displayed equivalent efficacy and greater safety than conventional ADCs and therefore have an improved therapeutic window, further highlighting the benefits of site-specific conjugation.[43]

Unnatural amino acids and selenocysteine

A second strategy for site-specific conjugation centers on the insertion of amino acids with bio-orthogonal reactive handles such as the twenty-first amino acid, selenocysteine, and the unnatural amino acid, acetylphenylalanine (pAcPhe). Two methods have been developed to employ these amino acids and both utilize stop codons, but one incorporates selenocysteine (Sec) by pairing the opal stop codon, UGA, with a Sec insertion sequence and the other incorporates acetylphenylalanine at the amber stop codon, UAG, using a tRNA/aminoacyl-tRNA synthetase pair.

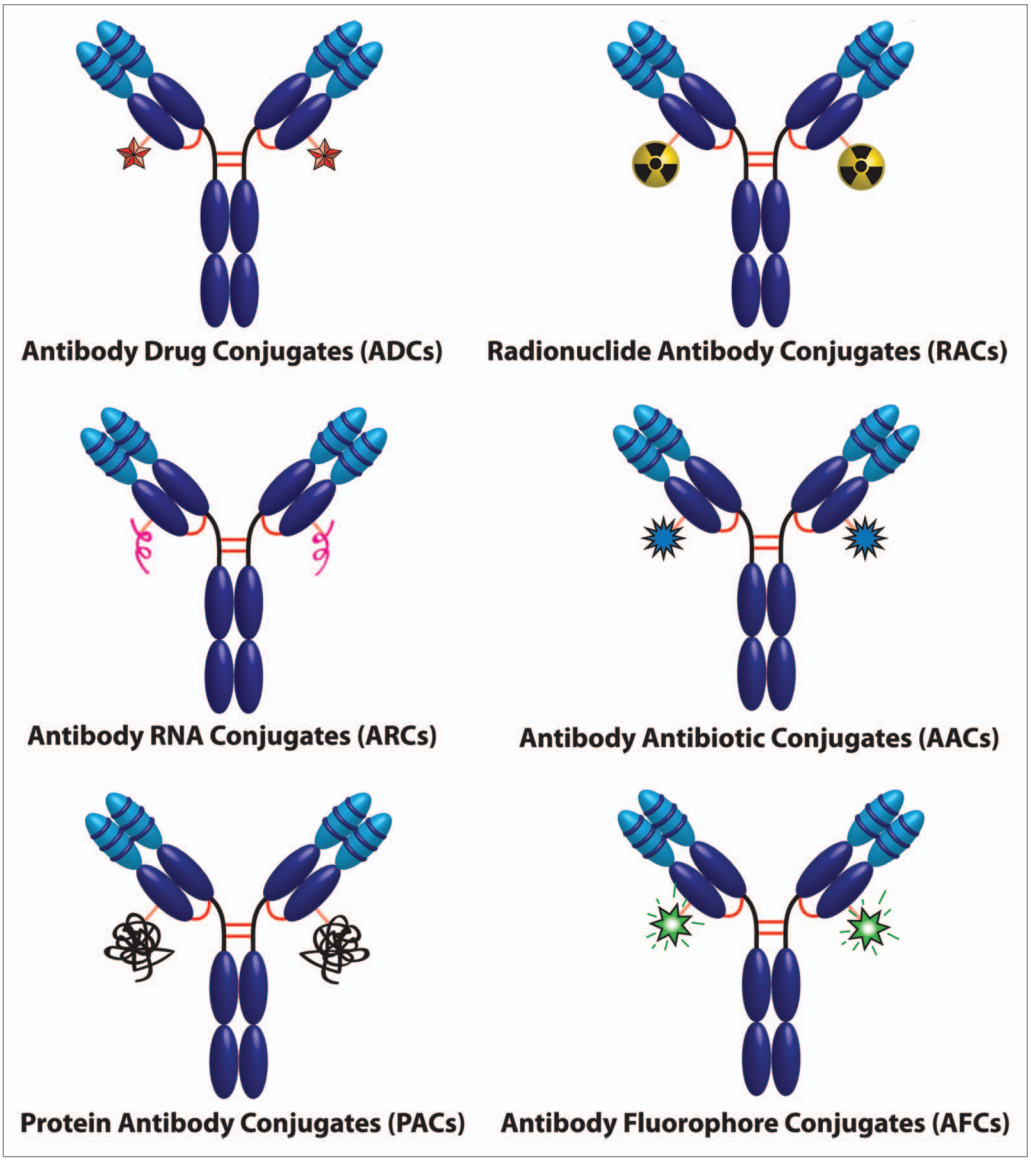

Figure 6. Applications of site-specific antibody conjugates. The site-specific conjugation of molecules to monoclonal antibodies has a wide range of applications. Site-specific conjugation decreases conjugate heterogeneity and improves stability and function. A number of possible antibody conjugates are represented here and include antibody-drug conjugates (ADCs) for cancer treatment, radionuclide-antibody conjugates (RACs) for imaging, antibody-antibiotic conjugates (AACs) to fight infectious disease, and antibody fluorophore conjugates (AFCs) for imaging and detection.

Selenocysteine, employed by the first method, is very similar to the classical amino acid, cysteine, but contains a selenium atom in place of the sulfur atom. The selenolate group is a more reactive nucleophile than the thiolate counterpart, rendering it amenable to conjugation with electrophilic compounds under conditions in which selenocysteine is selectively activated. There are approximately 25 known selenium-containing proteins in mammals, including proteins such as glutathione peroxidases and thioreductases.[51] Under normal conditions, UGA codes for transcriptional termination; however, in the presence of a Sec insertion sequence (SECIS) located in the 3' UTR of Sec containing proteins, termination is prevented by the formation of an mRNA secondary structure and Sec is inserted at the UGA codon.[52] Sec insertion can be engineered into non-Sec coding genes by insertion of the UGA codon and a SECIS at the 3' end of the gene. This technique was recently used in the Sec labeling and subsequent site-specific conjugation of mAbs.[53]

A second method for site-specific conjugation utilizes the unnatural amino acid, p-acetylphenylalanine (pAcPhe). pAcPhe contains a keto group that can be selectively conjugated to a drug containing an alkoxy-amine through an oxime ligation. To incorporate pAcPhe into an antibody, the amber stop codon is substituted into the antibody at the desired location. The antibody cDNA is then co-expressed with an amber suppressor tRNA and the properly paired mutant tRNA sythetase. The tRNA sythetase loads pAcPhe onto the amber tRNA and thus pAcPhe is incorporated into the antibody at the amber site UAG.[54,55] To

test the feasibility of using this concept with site-specific ADC conjugation, the amber stop codon was substituted for an alanine residue (A114) on the heavy-chain of the full-length anti-Her2 IgG gene of trastuzumab, the same conjugation site identified and described in the engineering of THIOMABs. The anti-Her2 IgG containing pAcPhe was then successfully produced by expressing it in a Chinese hamster ovary cell line along with the correct amber suppressor tRNA/aminoacyl-RNA synthetase pair. A linker with an alkoxy-amine was attached to the cytotoxic drug, auristatin F, and then conjugated to the pAcPhe anti-Her2 IgG. Conjugates were active in vivo and demonstrated the feasibility of using unnatural amino acids to generate site-specific ADCs.[56]

In addition to the example of pAcPhe described above, other unnatural amino acids are also under investigation for use in site-specific conjugation using similar processes involving matching tRNA/aminoacyl-tRNA synthetase pairs.[57,58] In vitro transcription and translation methods were recently developed for the expression of antibodies and can likely be tailored for the site-specific incorporation of unnatural amino acids.[59]

Enzymatic conjugation: glycotransferases and transglutaminases

The use of enzymes to catalyze bond formation is another strategy being explored for use in site-specific conjugation. Two platforms, one based on glycotransferases and a second based on transglutaminases, were recently developed and appear promising. The glycotransferase platform uses a mutant glycotransferase to attach a chemically active sugar moiety to a glycosylation site on an antibody. Molecules of choice can then be conjugated to the chemical handle on the sugar moiety. In the second platform, transglutaminase is used to form a bond between an amine group on the linker/drug and an engineered glutamine residue on the antibody. Both platforms are being investigated for the production of ADCs and are discussed in greater detail below.

Glycotransferases are a large family of proteins involved in the synthesis of oligosaccharides and are responsible for the transfer of a sugar residue from an activated sugar nucleotide to a sugar acceptor or glycoprotein/lipid. The structures of several glycotransferases are known and reveal that sugar donor specificity is determined by a few amino acids in the catalytic pocket.[60] Using this knowledge, residues were mutated in the pocket of the glycotransferase, B4Gal-T1, to broaden donor specificity and allow the transfer of the chemically reactive sugar residue, 2-keto-Gal.[61] This technology allows for the ability to transfer a chemically reactive sugar to any lipid or protein containing a glycosylation site.

Human IgG antibodies contain an N-glycosylation site at the conserved Asn-297 of the Fc fragment. The glycans attached to this site are generally complex, but can be degalactosylated down to G0, onto which a mutant glycotransferase is capable of transferring C2-keto-Gal with high efficiency.[62] The active chemical handle of C2-keto Gal can then be coupled to biomolecules with an orthogonal reactive group. This approach was used successfully for the site-specific conjugation of the anti-Her2 antibody, trastuzumab, with Alexa Fluor 488 aminooxyacetamide and should be a viable technique for site-specific ADC generation.[62]

The second platform utilizes transglutaminase to catalyze the formation of a covalent bond between a free amine group and a glutamine side chain. Transglutaminase from *Streptoverticillium mobaraense* (mTG) is commercially available and has been used extensively as a protein crosslinking agent.[63] mTG does not recognize any of the natural occurring glutamine residues in the Fc region of glycosylated antibodies, but does recognize a "glutamine tag" that can be engineered into an antibody.[64] The glutamine tag, LLQG, was engineered into different sites in the constant domain of an antibody targeting the epidermal growth factor receptor. mTG was then used to conjugate these sites with fluorophores or monomethyl dolastatin 10 (MMAD) and several sites where found to have good biophysical properties and a high degree of conjugation. mTG was also able to conjugate to glutamine tags on anti-Her2 and anti-M1S1 antibodies. An anti-M1S1-vc-MMAD conjugate displayed strong in vitro and in vivo activity, suggesting that conjugation using this method does not alter antibody binding or affinity and demonstrates the utility of this approach in the site-specific conjugation of ADCs.[65]

In addition to glycotransferases and transglutaminases, other enzymes have been explored for use in protein labeling.[66] One such enzyme, formylglycine generating enzyme, recognizes the sequence CxPxR and oxidizes a cysteine residue to form formylglycine, thus generating a protein with an aldehyde tag. The aldehyde group can then be conjugated to molecule of choice through hydrozino-Pictet-Spengler chemistry. This technique appears promising and is under investigation for use in the site-specific labeling of antibodies.[67,68]

Applications of Site-Specific Antibody Conjugates

MAbs are of great use in many applications ranging from basic research to treatment of disease. The ability to conjugate a wide variety of molecules to mAbs has increased their functionality even further. Traditional conjugation is performed by attaching molecules to reactive lysine or cysteine residues on antibodies. However, conjugation using these approaches can occur at a number of different sites and to a varying degree, resulting in large heterogeneity of conjugate species. Site-specific conjugation has emerged as a way to decrease heterogeneity and improve antibody conjugate consistency and functionality.

A number of site-specific conjugation methods are currently under investigation and five methods were described in detail in previous sections. All of these methods result in site-specific conjugation, but several differences between the methods exist, including the requirement for genetic modification of antibodies, use of enzymes for conjugation, and conjugation site number and/or location (**Table 1**).

As discussed in detail above, ADC development benefits greatly from site-specific conjugation because of the improvement in manufacturing heterogeneity and increase in therapeutic window. Recently, the site-specific approach has also allowed in-depth study of how the conjugation site modulates in vivo ADC stability and therapeutic activity.[50] In this study, engineered cysteine technology was used to generate three different trastuzumab THIOMABs, one with a highly accessible

conjugation site (Fc-S396C), one with a partially buried site in a positively charged environment (LC-V205C), and one with a partially buried site in a neutral environment (HC-A114C). The cytotoxic drug, monomethyl auristatin E (MMAE), was conjugated to the three trastuzumab variants using a protease cleavable linker and in vivo therapeutic efficacy was determined.[50] Despite a similar drug load and affinity, the three variants displayed different therapeutic activity. This variable activity was due to in vivo linker stability resulting from a difference in the structural and chemical environments surrounding the conjugation sites. The highly solvent-accessible site allowed maleimide exchange of the linker-drug with albumin, cysteine, or reduced glutathione in the plasma. The conjugate with the greatest therapeutic activity contained the partially buried thiol site in a positively charged environment, which allowed succinimide ring hydrolysis, prevented maleimide exchange and improved conjugate stability.[50] This important discovery would not have been possible without site-specific conjugation.

Another application of site-specific conjugation is the generation of Radionuclide Antibody Conjugates (RACs) for use as therapeutics or imaging agents. There are currently two marketed RACs, ibritumomab tiuxetan (Zevalin®) and tositumomab (Bexxar®), for the treatment of lymphoma, in which radionuclides are targeted to tumors by anti-CD20 mAbs.[69] Both of these molecules are generated through conventional conjugation, but they and future therapeutic RACs would likely benefit from the use of site-specific conjugation. RACs can also be used in methods such as Immuno-positron emission tomography (ImmunoPET or iPET), to track and quantify antibodies in vivo or for diagnostic purposes. For example, engineered cysteine residues were used to generate trastuzumab THIOMABs, which were subsequently labeled with ^{89}Zr. These trastuzumab THIOMAB ^{89}Zr RACs were then used successfully in ImmunoPET experiments to track in vivo conjugate distribution and tumor uptake.[70]

Antibodies can also be conjugated to many other molecules for research and therapeutics applications. Current antibody conjugates include Antibody RNA Conjugates (ARCs) for delivery of siRNAs,[71,72] Antibody Antibiotic Conjugates (AACs) to target pathogens,[73,74] Antibody Fluorophore Conjugates (AFCs) for imaging and laboratory reagents,[50] and Protein Antibody Conjugates (PACs) for the treatment of cancer.[75-77] Site-specific conjugation can be explored for use with these conjugates and would likely improve their production, stability, and homogeneity (**Fig. 6**). The development of methodologies for site-specific conjugation has expanded the utility of mAbs into many exciting future applications, ensuring a significant position for these powerful molecules at the forefront of research and therapeutics.

Disclosure of Potential Conflicts of Interest

All authors are full time employees of Genentech, Inc.

References

1. Shuptrine CW, Surana R, Weiner LM. Monoclonal antibodies for the treatment of cancer. Semin Cancer Biol 2012; 22:3-13; PMID:22245472; http://dx.doi.org/10.1016/j.semcancer.2011.12.009
2. Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. Monoclonal antibody successes in the clinic. Nat Biotechnol 2005; 23:1073-8; PMID:16151394; http://dx.doi.org/10.1038/nbt0905-1073
3. Kubota T, Niwa R, Satoh M, Akinaga S, Shitara K, Hanai N. Engineered therapeutic antibodies with improved effector functions. Cancer Sci 2009; 100:1566-72; PMID:19538497; http://dx.doi.org/10.1111/j.1349-7006.2009.01222.x
4. Lambert JM. Drug-conjugated monoclonal antibodies for the treatment of cancer. Curr Opin Pharmacol 2005; 5:543-9; PMID:16087399; http://dx.doi.org/10.1016/j.coph.2005.04.017
5. Senter PD. Potent antibody drug conjugates for cancer therapy. Curr Opin Chem Biol 2009; 13:235-44; PMID:19414278; http://dx.doi.org/10.1016/j.cbpa.2009.03.023
6. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol 2005; 23:1137-46; PMID:16151407; http://dx.doi.org/10.1038/nbt1141
7. DeVita VT Jr., Chu E. A history of cancer chemotherapy. Cancer Res 2008; 68:8643-53; PMID:18974103; http://dx.doi.org/10.1158/0008-5472.CAN-07-6611
8. Schwartz RS. Paul Ehrlich's magic bullets. N Engl J Med 2004; 350:1079-80; PMID:15014180; http://dx.doi.org/10.1056/NEJMp048021
9. Trail PA, Willner D, Lasch SJ, Henderson AJ, Hofstead S, Casazza AM, Firestone RA, Hellström I, Hellström KE. Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science 1993; 261:212-5; PMID:8327892; http://dx.doi.org/10.1126/science.8327892
10. Varki N, Reisfeld R, Walter L. Effects of monoclonal antibody-drug conjugates on the in vivo growth of human tumors established in nude mice. New York: Alan R. Liss Inc, 1985.
11. Elias DJ, Hirschowitz L, Kline LE, Kroener JF, Dillman RO, Walker LE, Robb JA, Timms RM. Phase I clinical comparative study of monoclonal antibody KS1/4 and KS1/4-methotrexate immunconjugate in patients with non-small cell lung carcinoma. Cancer Res 1990; 50:4154-9; PMID:2162255
12. Saleh MN, Sugarman S, Murray J, Ostroff JB, Healey D, Jones D, et al. Phase I trial of the anti-Lewis Y drug immunoconjugate BR96-doxorubicin in patients with lewis Y-expressing epithelial tumors. Journal of clinical oncology: official journal of the American Society of Clinical Oncology 2000; 18:2282-92.
13. Tolcher AW, Sugarman S, Gelmon KA, Cohen R, Saleh M, Isaacs C, et al. Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology 1999; 17:478-84.
14. Sievers EL, Linenberger M. Mylotarg: antibody-targeted chemotherapy comes of age. Curr Opin Oncol 2001; 13:522-7; PMID:11673694; http://dx.doi.org/10.1097/00001622-200111000-00016
15. Ravandi F. Gemtuzumab ozogamicin: one size does not fit all–the case for personalized therapy. Journal of clinical oncology: official journal of the American Society of Clinical Oncology 2011; 29:349-51.
16. Petersdorf S, Kopecky K, Stuart R. Preliminary results of Southwest Oncology Group Study S0106: An international intergroup phase 3 randomized trial comparing the addition of gemtuzumab ozogamicin to standard induction therapy versus standard induction therapy followed by a second randomization to post-consolidation gemtuzumab ozogamicin versus no additional therapy for previously untreated acute myeloid leukemia. [abstract 790]. Blood 2009; 114:326
17. Senter PD, Sievers EL. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. Nat Biotechnol 2012; 30:631-7; PMID:22781692; http://dx.doi.org/10.1038/nbt.2289
18. Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, Blättler WA, Lambert JM, Chari RV, Lutz RJ, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res 2008; 68:9280-90; PMID:19010901; http://dx.doi.org/10.1158/0008-5472.CAN-08-1776
19. Ritchie M, Tchistiakova L, Scott N. Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates. MAbs 2013; 5:13-21; PMID:23221464; http://dx.doi.org/10.4161/mabs.22854
20. Sievers EL, Senter PD. Antibody-drug conjugates in cancer therapy. Annu Rev Med 2013; 64:15-29; PMID:23043493; http://dx.doi.org/10.1146/annurev-med-050311-201823
21. Kovtun YV, Audette CA, Ye Y, Xie H, Ruberti MF, Phinney SJ, Leece BA, Chittenden T, Blättler WA, Goldmacher VS. Antibody-drug conjugates designed to eradicate tumors with homogeneous and heterogeneous expression of the target antigen. Cancer Res 2006; 66:3214-21; PMID:16540673; http://dx.doi.org/10.1158/0008-5472.CAN-05-3973
22. Teicher BA. Antibody-drug conjugate targets. Curr Cancer Drug Targets 2009; 9:982-1004; PMID:20025606; http://dx.doi.org/10.2174/156800909790192365
23. Chari RV. Targeted cancer therapy: conferring specificity to cytotoxic drugs. Acc Chem Res 2008; 41:98-107; PMID:17705444; http://dx.doi.org/10.1021/ar700108g

24. Alley SC, Zhang X, Okeley NM, Anderson M, Law CL, Senter PD, Benjamin DR. The pharmacologic basis for antibody-auristatin conjugate activity. J Pharmacol Exp Ther 2009; 330:932-8; PMID:19498104; http://dx.doi.org/10.1124/jpet.109.155549
25. Polson AG, Williams M, Gray AM, Fuji RN, Poon KA, McBride J, Raab H, Januario T, Go M, Lau J, et al. Anti-CD22-MCC-DM1: an antibody-drug conjugate with a stable linker for the treatment of non-Hodgkin's lymphoma. Leukemia 2010; 24:1566-73; PMID:20596033; http://dx.doi.org/10.1038/leu.2010.141
26. Carter PJ, Senter PD. Antibody-drug conjugates for cancer therapy. Cancer J 2008; 14:154-69; PMID:18536555; http://dx.doi.org/10.1097/PPO.0b013e318172d704
27. Ducry L, Stump B. Antibody-drug conjugates: linking cytotoxic payloads to monoclonal antibodies. Bioconjug Chem 2010; 21:5-13; PMID:19769391; http://dx.doi.org/10.1021/bc9002019
28. Polson AG, Calemine-Fenaux J, Chan P, Chang W, Christensen E, Clark S, de Sauvage FJ, Eaton D, Elkins K, Elliott JM, et al. Antibody-drug conjugates for the treatment of non-Hodgkin's lymphoma: target and linker-drug selection. Cancer Res 2009; 69:2358-64; PMID:19258515; http://dx.doi.org/10.1158/0008-5472.CAN-08-2250
29. Sedlacek H-H, Seemean G, Hoffmann D, Czech J, Lorenz P. Antibodies as carriers of cytotoxicity. Contributions to Oncology 1992; 43:1-145
30. Moldenhauer G, Salnikov AV, Lüttgau S, Herr I, Anderl J, Faulstich H. Therapeutic potential of amanitin-conjugated anti-epithelial cell adhesion molecule monoclonal antibody against pancreatic carcinoma. J Natl Cancer Inst 2012; 104:622-34; PMID:22457476; http://dx.doi.org/10.1093/jnci/djs140
31. Doronina SO, Toki BE, Torgov MY, Mendelsohn BA, Cerveny CG, Chace DF, DeBlanc RL, Gearing RP, Bovee TD, Siegall CB, et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nat Biotechnol 2003; 21:778-84; PMID:12778055; http://dx.doi.org/10.1038/nbt832
32. Gerber HP, Kung-Sutherland M, Stone I, Morris-Tilden C, Miyamoto J, McCormick R, Alley SC, Okeley N, Hayes B, Hernandez-Ilizaliturri FJ, et al. Potent antitumor activity of the anti-CD19 auristatin antibody drug conjugate hBU12-vcMMAE against rituximab-sensitive and -resistant lymphomas. Blood 2009; 113:4352-61; PMID:19147785; http://dx.doi.org/10.1182/blood-2008-09-179143
33. Kupchan SM, Komoda Y, Court WA, Thomas GJ, Smith RM, Karim A, Gilmore CJ, Haltiwanger RC, Bryan RF. Maytansine, a novel antileukemic ansa macrolide from Maytenus ovatus. J Am Chem Soc 1972; 94:1354-6; PMID:5062169; http://dx.doi.org/10.1021/ja00759a054
34. Remillard S, Rebhun LI, Howie GA, Kupchan SM. Antimitotic activity of the potent tumor inhibitor maytansine. Science 1975; 189:1002-5; PMID:1241159; http://dx.doi.org/10.1126/science.1241159
35. Boger DL, Patane MA, Jin Q, Kitos PA. Design, synthesis and evaluation of bouvardin, deoxybouvardin and RA-I-XIV pharmacophore analogs. Bioorg Med Chem 1994; 2:85-100; PMID:7922127; http://dx.doi.org/10.1016/S0968-0896(00)82005-2
36. Thorson JS, Sievers EL, Ahlert J, Shepard E, Whitwam RE, Onwueme KC, Ruppen M. Understanding and exploiting nature's chemical arsenal: the past, present and future of calicheamicin research. Curr Pharm Des 2000; 6:1841-79; PMID:11102565; http://dx.doi.org/10.2174/1381612003398564
37. Boger DL, Johnson DS. CC-1065 and the duocarmycins: unraveling the keys to a new class of naturally derived DNA alkylating agents. Proc Natl Acad Sci U S A 1995; 92:3642-9; PMID:7731958; http://dx.doi.org/10.1073/pnas.92.9.3642
38. Hartley JA. The development of pyrrolobenzodiazepines as antitumour agents. Expert Opin Investig Drugs 2011; 20:733-44; PMID:21457108; http://dx.doi.org/10.1517/13543784.2011.573477
39. Wang L, Amphlett G, Blattler WA, Lambert JM, Zhang W. Structural characterization of the maytansinoid-monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. Protein science: a publication of the Protein Society 2005; 14:2436-46.
40. Junutula JR, Flagella KM, Graham RA, Parsons KL, Ha E, Raab H, et al. Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. Clinical cancer research: an official journal of the American Association for Cancer Research 2010; 16:4769-78.
41. Dere R, Yi JH, Lei C, Saad OM, Huang C, Li Y, Baudys J, Kaur S. PK assays for antibody-drug conjugates: case study with ado-trastuzumab emtansine. Bioanalysis 2013; 5:1025-40; PMID:23641694; http://dx.doi.org/10.4155/bio.13.72
42. Hamblett KJ, Senter PD, Chace DF, Sun MM, Lenox J, Cerveny CG, et al. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clinical cancer research: an official journal of the American Association for Cancer Research 2004; 10:7063-70.
43. Junutula JR, Raab H, Clark S, Bhakta S, Leipold DD, Weir S, Chen Y, Simpson M, Tsai SP, Dennis MS, et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat Biotechnol 2008; 26:925-32; PMID:18641636; http://dx.doi.org/10.1038/nbt.1480
44. McDonagh CF, Turcott E, Westendorf L, Webster JB, Alley SC, Kim K, Andreyka J, Stone I, Hamblett KJ, Francisco JA, et al. Engineered antibody-drug conjugates with defined sites and stoichiometries of drug attachment. Protein Eng Des Sel 2006; 19:299-307; PMID:16644914; http://dx.doi.org/10.1093/protein/gzl013
45. Woo HJ, Lotz MM, Jung JU, Mercurio AM. Carbohydrate-binding protein 35 (Mac-2), a laminin-binding lectin, forms functional dimers using cysteine 186. J Biol Chem 1991; 266:18419-22; PMID:1917966
46. Wootton SK, Yoo D. Homo-oligomerization of the porcine reproductive and respiratory syndrome virus nucleocapsid protein and the role of disulfide linkages. J Virol 2003; 77:4546-57; PMID:12663761; http://dx.doi.org/10.1128/JVI.77.8.4546-4557.2003
47. Junutula JR, Bhakta S, Raab H, Ervin KE, Eigenbrot C, Vandlen R, Scheller RH, Lowman HB. Rapid identification of reactive cysteine residues for site-specific labeling of antibody-Fabs. J Immunol Methods 2008; 332:41-52; PMID:18230399; http://dx.doi.org/10.1016/j.jim.2007.12.011
48. Boswell CA, Mundo EE, Zhang C, Bumbaca D, Valle NR, Kozak KR, Fourie A, Chuh J, Koppada N, Saad O, et al. Impact of drug conjugation on pharmacokinetics and tissue distribution of anti-STEAP1 antibody-drug conjugates in rats. Bioconjug Chem 2011; 22:1994-2004; PMID:21913715; http://dx.doi.org/10.1021/bc200212a
49. Boswell CA, Mundo EE, Zhang C, Stainton SL, Yu SF, Lacap JA, et al. Differential effects of predosing on tumor and tissue uptake of an 111In-labeled anti-TENB2 antibody-drug conjugate. Journal of nuclear medicine: official publication, Society of Nuclear Medicine 2012; 53:1454-61.
50. Shen BQ, Xu K, Liu L, Raab H, Bhakta S, Kenrick M, Parsons-Reponte KL, Tien J, Yu SF, Mai E, et al. Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. Nat Biotechnol 2012; 30:184-9; PMID:22267010; http://dx.doi.org/10.1038/nbt.2108
51. Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigó R, Gladyshev VN. Characterization of mammalian selenoproteomes. Science 2003; 300:1439-43; PMID:12775843; http://dx.doi.org/10.1126/science.1083516
52. Caban K, Copeland PR. Size matters: a view of selenocysteine incorporation from the ribosome. Cell Mol Life Sci 2006; 63:73-81; PMID:16416259; http://dx.doi.org/10.1007/s00018-005-5402-y
53. Hofer T, Skeffington LR, Chapman CM, Rader C. Molecularly defined antibody conjugation through a selenocysteine interface. Biochemistry 2009; 48:12047-57; PMID:19894757; http://dx.doi.org/10.1021/bi901744t
54. Liu W, Brock A, Chen S, Chen S, Schultz PG. Genetic incorporation of unnatural amino acids into proteins in mammalian cells. Nat Methods 2007; 4:239-44; PMID:17322890; http://dx.doi.org/10.1038/nmeth1016
55. Wang L, Zhang Z, Brock A, Schultz PG. Addition of the keto functional group to the genetic code of Escherichia coli. Proc Natl Acad Sci U S A 2003; 100:56-61; PMID:12518054; http://dx.doi.org/10.1073/pnas.0234824100
56. Axup JY, Bajjuri KM, Ritland M, Hutchins BM, Kim CH, Kazane SA, Halder R, Forsyth JS, Santidrian AF, Stafin K, et al. Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. Proc Natl Acad Sci U S A 2012; 109:16101-6; PMID:22988081; http://dx.doi.org/10.1073/pnas.1211023109
57. Young TS, Ahmad I, Yin JA, Schultz PG. An enhanced system for unnatural amino acid mutagenesis in E. coli. J Mol Biol 2010; 395:361-74; PMID:19852970; http://dx.doi.org/10.1016/j.jmb.2009.10.030
58. Kiick KL, Saxon E, Tirrell DA, Bertozzi CR. Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. Proc Natl Acad Sci U S A 2002; 99:19-24; PMID:11752401; http://dx.doi.org/10.1073/pnas.012583299
59. Yin G, Garces ED, Yang J, Zhang J, Tran C, Steiner AR, et al. Aglycosylated antibodies and antibody fragments produced in a scalable in vitro transcription-translation system. MAbs 2012; 4; PMID:22377750
60. Qasba PK, Ramakrishnan B, Boeggeman E. Substrate-induced conformational changes in glycosyltransferases. Trends Biochem Sci 2005; 30:53-62; PMID:15653326; http://dx.doi.org/10.1016/j.tibs.2004.11.005
61. Ramakrishnan B, Qasba PK. Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. J Biol Chem 2002; 277:20833-9; PMID:11916963; http://dx.doi.org/10.1074/jbc.M111183200
62. Boeggeman E, Ramakrishnan B, Pasek M, Manzoni M, Puri A, Loomis KH, Waybright TJ, Qasba PK. Site specific conjugation of fluoroprobes to the remodeled Fc N-glycans of monoclonal antibodies using mutant glycosyltransferases: application for cell surface antigen detection. Bioconjug Chem 2009; 20:1228-36; PMID:19425533; http://dx.doi.org/10.1021/bc900103p
63. Yokoyama K, Nio N, Kikuchi Y. Properties and applications of microbial transglutaminase. Appl Microbiol Biotechnol 2004; 64:447-54; PMID:14740191; http://dx.doi.org/10.1007/s00253-003-1539-5
64. Jeger S, Zimmermann K, Blanc A, Grünberg J, Honer M, Hunziker P, Struthers H, Schibli R. Site-specific and stoichiometric modification of antibodies by bacterial transglutaminase. Angew Chem Int Ed Engl 2010; 49:9995-7; PMID:21110357; http://dx.doi.org/10.1002/anie.201004243

65. Strop P, Liu SH, Dorywalska M, Delaria K, Dushin RG, Tran TT, Ho WH, Farias S, Casas MG, Abdiche Y, et al. Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. Chem Biol 2013; 20:161-7; PMID:23438745; http://dx.doi.org/10.1016/j.chembiol.2013.01.010
66. Sunbul M, Yin J. Site specific protein labeling by enzymatic posttranslational modification. Org Biomol Chem 2009; 7:3361-71; PMID:19675886; http://dx.doi.org/10.1039/b908687k
67. Carrico IS, Carlson BL, Bertozzi CR. Introducing genetically encoded aldehydes into proteins. Nat Chem Biol 2007; 3:321-2; PMID:17450134; http://dx.doi.org/10.1038/nchembio878
68. Rabuka D, Rush JS, deHart GW, Wu P, Bertozzi CR. Site-specific chemical protein conjugation using genetically encoded aldehyde tags. Nat Protoc 2012; 7:1052-67; PMID:22576105; http://dx.doi.org/10.1038/nprot.2012.045
69. Steiner M, Neri D. Antibody-radionuclide conjugates for cancer therapy: historical considerations and new trends. Clinical cancer research: an official journal of the American Association for Cancer Research 2011; 17:6406-16.
70. Tinianow JN, Gill HS, Ogasawara A, Flores JE, Vanderbilt AN, Luis E, Vandlen R, Darwish M, Junutula JR, Williams SP, et al. Site-specifically 89Zr-labeled monoclonal antibodies for ImmunoPET. Nucl Med Biol 2010; 37:289-97; PMID:20346868; http://dx.doi.org/10.1016/j.nucmedbio.2009.11.010
71. Pardridge WM. shRNA and siRNA delivery to the brain. Adv Drug Deliv Rev 2007; 59:141-52; PMID:17434235; http://dx.doi.org/10.1016/j.addr.2007.03.008
72. Tan M, Vernes JM, Chan J, Cuellar TL, Asundi A, Nelson C, Yip V, Shen B, Vandlen R, Siebel C, et al. Real-time quantification of antibody-short interfering RNA conjugate in serum by antigen capture reverse transcription-polymerase chain reaction. Anal Biochem 2012; 430:171-8; PMID:22929697; http://dx.doi.org/10.1016/j.ab.2012.08.018
73. Franzman MR, Burnell KK, Dehkordi-Vakil FH, Guthmiller JM, Dawson DV, Brogden KA. Targeted antimicrobial activity of a specific IgG-SMAP28 conjugate against Porphyromonas gingivalis in a mixed culture. Int J Antimicrob Agents 2009; 33:14-20; PMID:18778918; http://dx.doi.org/10.1016/j.ijantimicag.2008.05.021
74. Kim SA. Antibiotic-conjugated antibodies. United States patent US 7,569,677 B2: Strox Biopharmaceuticals, 2009.
75. Gillies SD, Young D, Lo KM, Foley SF, Reisfeld RA. Expression of genetically engineered immunoconjugates of lymphotoxin and a chimeric anti-ganglioside GD2 antibody. Hybridoma 1991; 10:347-56; PMID:1916847; http://dx.doi.org/10.1089/hyb.1991.10.347
76. Hoogenboom HR, Volckaert G, Raus JC. Construction and expression of antibody-tumor necrosis factor fusion proteins. Mol Immunol 1991; 28:1027-37; PMID:1922108; http://dx.doi.org/10.1016/0161-5890(91)90189-Q
77. Ronca R, Sozzani S, Presta M, Alessi P. Delivering cytokines at tumor site: The immunocytokine-conjugated anti-EDB-fibronectin antibody case. Immunobiology 2009; 214:800-10; PMID:19625102; http://dx.doi.org/10.1016/j.imbio.2009.06.005