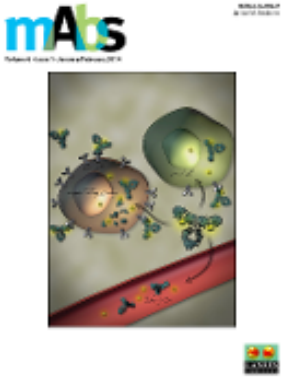

mAbs

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/kmab20

Site-specific antibody drug conjugates for cancer therapy

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Published online: 01 Nov 2013.

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

Site-specific antibody drug conjugates for cancer therapy

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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

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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.

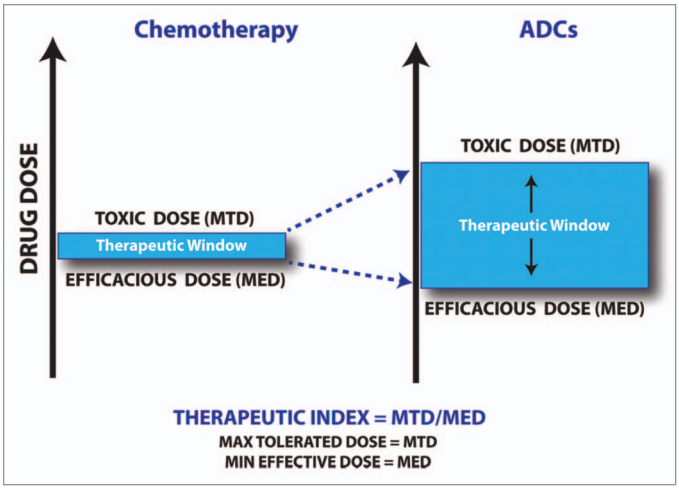

Figure 1. ADCs expand the therapeutic window. ADC therapeutics can increase efficacy and decrease toxicity in comparison to traditional chemotherapeutic cancer treatments. Select delivery of drugs to cancer cells increases the percent of dosed drug reaching the tumor, thus lowering the minimum effective 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

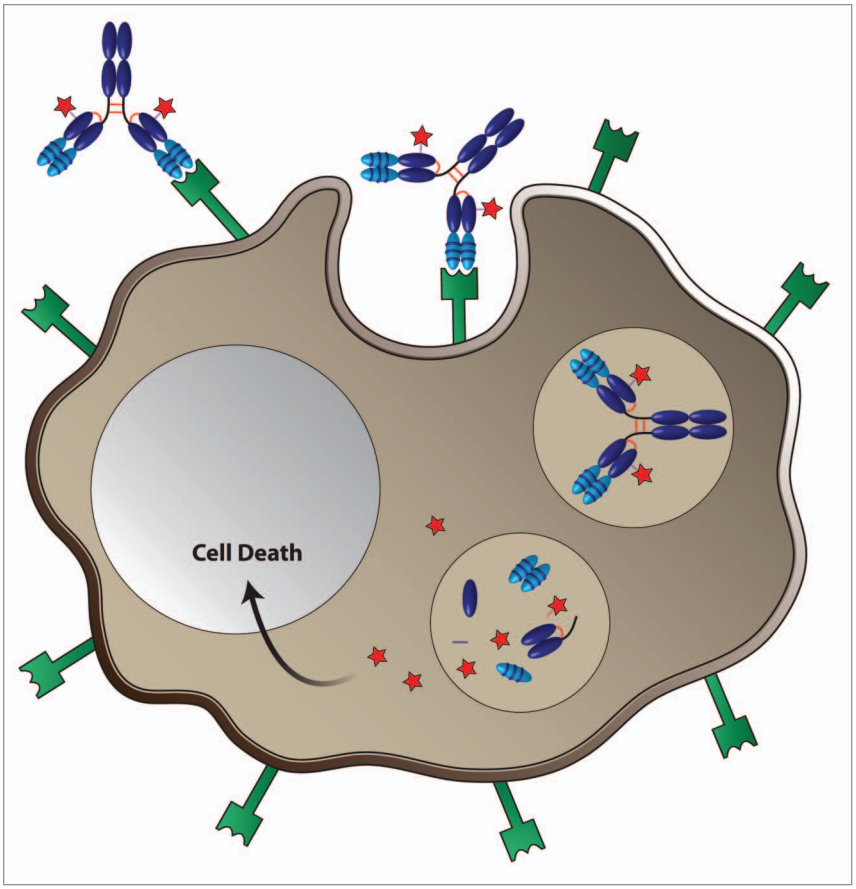

Figure 2. Delivery of cytotoxic drugs to cancer cells by ADCs. The monoclonal antibody component of an ADC selectively binds a cell-surface tumor antigen, resulting in internalization of the ADC-antigen complex through the process of receptor-mediated endocytosis. The ADC-antigen complex then traffics 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.

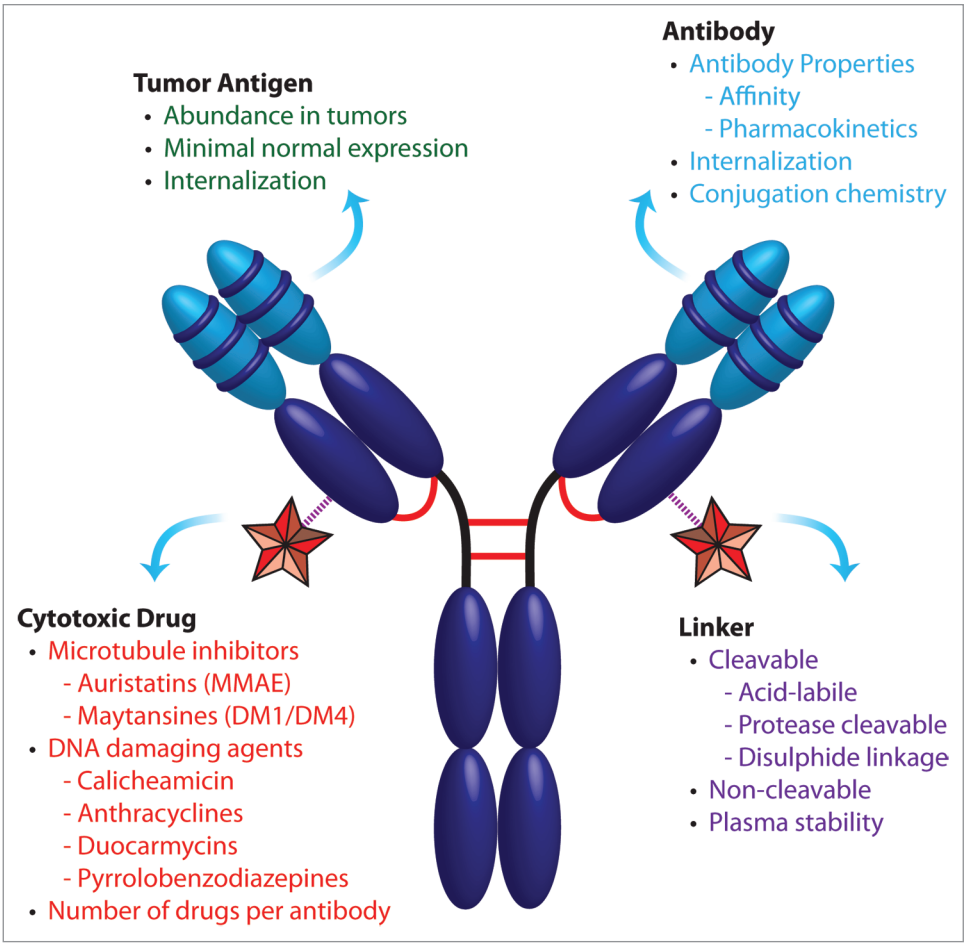

Figure 3. Critical factors that influence ADC therapeutics. ADCs consist of a cytotoxic drug conjugated to a monoclonal antibody by means of a select linker. These components all affect ADC performance and their optimization is essential for development of successful conjugates.

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.

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