Expert Opinion

Antibody–cytotoxic agent conjugates for cancer therapy

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Antibody-based delivery of cytotoxic agents, including toxins, to tumours can dramatically reduce systemic toxicity and increase therapeutic efficacy. The advantage of a monoclonal antibody (mAb) is superior selectivity towards antigens expressed on the surface of cancer cells. Recent advances in biotechnology accelerated progress in the pharmaceutical applications of mAbs. A cytotoxic warhead is attached to a mAb in an immunoconjugate via a linker, which is stable in circulation but efficiently cleaved in the tumour tissue. The warhead, mAb and linker play important roles in the successful design of potent and efficient immunoconjugates. To date, one mAb–cytotoxic agent conjugate has been approved by the FDA and several other candidates are in various stages of clinical trials. This review describes the recent progress in the design and development of mAb-based immunoconjugates of cytotoxic agents, and summarises the criteria for the critical choices of a suitable mAb, linker and cytotoxic agent to design an efficacious immunoconjugate.

Keywords: cancer therapy, cytotoxic agent, mAb-based immunoconjugate, monoclonal antibody, toxin, tumour targeting

Expert Opin. Drug Deliv. (2005) 2(5):873-890

1. Introduction

Cancer is the second leading cause of death in the US, and remains one of the most challenging diseases for physicians and biomedical scientists in academia and the pharmaceutical industry to combat. One of the major reasons for difficulty in cancer treatment is that, unlike bacteria or viruses, cancer cells originate from the host. As a result, cancer cells do not contain molecular targets that are completely foreign to the host. Traditional cancer chemotherapy is based on the principle that rapidly proliferating tumour cells are more likely to be killed by cytotoxic drugs. Unfortunately, the current cytotoxic chemotherapeutic agents, such as paclitaxel, cisplatin, doxorubicin and other widely used anticancer drugs, lack the specificity required to kill tumour cells without simultaneously damaging healthy tissue and thus causing severe side effects [1]. Consequently, patients would be at risk if exposed to the high doses of cytotoxic agents that are required to eradicate the tumour completely. This is a particularly serious problem for the treatment of a solid tumour because most of these tumour cells are growing slowly.

In order to solve the tumour-specificity problem associated with chemotherapeutic agents, the development of new cytotoxic agents, or their prodrugs, with greater selectivity to tumours is an urgent need in cancer chemotherapy. One of the most promising strategies is the tumour-specific delivery of cytotoxic agents by recognising the differences between normal and cancer cells. It is conceivable that a prodrug with minimal systemic toxicity can be constructed by conjugating a cytotoxic agent to a tumour-targeting molecule. This prodrug is delivered to malignant tissue cells and internalised to release the cytotoxic agent to kill the cancer cells.

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Table 1. mAb–cytotoxic agent conjugates in clinic and clinical studies for cancer therapy.

Name	Cytotoxic agent	mAb	Target antigen	Target cancers	Stage
Gemtuzumab–ozogamicin [8]	Calicheamicin	huP67.6	CD33	Acute myeloid leukaemia	FDA approved
huC242–DM1 [9]	Maytansine	huC242	CanAg	Colorectal, pancreatic, gastric, NSCLC	Phase II
huN901–DM1 [10]	Maytansine	hu901	CD56	Small cell lung	Phase II
MLN2704–DM1 [10]	Maytansine	MLN591	PSMA	Prostate	Phase I
Trastuzumab–DM1 [10]	Maytansine	Trastuzumab	HER2	Breast	Phase I
Bivatuzumab–mertansine [10]	Maytansine	Anti-CD44v6	CD44v6	Colorectal, pancreatic	Phase I
BR96– doxorubicin [11]	Doxorubicin	BR96	Lewis Y	NSCLC, breast, colorectal, prostate, ovarian	Phase II
HCTM01–calicheamicin [12,13]	Calicheamicin	CTM01	MUC1	Ovarian	Phase II

mAb: Monoclonal antibody; NSCLC: Non-small cell lung cancer.

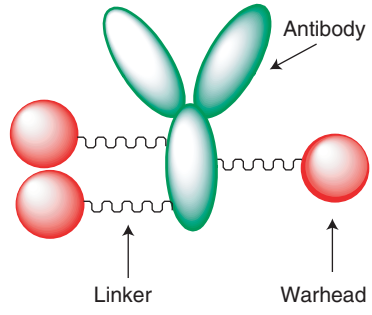


Figure 1. Antibody–cytotoxic agent conjugate.

The discovery of antigens that are particularly over-expressed on a cancer cell membrane and the first description of monoclonal antibodies (mAbs) possessing extremely high binding affinity to tumour-specific antigens opened a new era for the development of mAb-based tumour-targeting chemotherapy [2-4]. The mAb-based chemotherapy already has a long history. As early as 1956, Ehrlich [5,6] introduced the concept of using diphtheria toxin bound to antitumour antibodies to treat malignant diseases. This attempt was not successful due to the technical difficulty in obtaining appropriate antibodies. In 1958, Mathé et al. [7] produced an immunoconjugate composed of methotrexate and an antitumour antibody. This prodrug was successfully used to cure leukaemia in a mouse model.

Although the 50-year old idea is intriguing, it was only recently that some clinically successful immunoconjugates were identified. The application of novel highly cytotoxic agents, advances in mAb production and improvements in linker technologies accelerated the progress in mAb–cytotoxic agent conjugates in the past three decades. Gemtuzumab-ozogamicin (Mylotarg®), developed by Wyeth, is the first mAb–cytotoxic agent conjugate approved by the FDA in 2000 for the treatment of acute myelogenous leukaemia [8]. Several other immunoconjugates also showed impressive results and are currently in Phase I or II human clinical trials as listed in Table 1 [8-13].

This review describes the general concept for the design of mAb–cytotoxic agent conjugates and concisely summarises the advances in immunoconjugate technology in the last three decades. Individual components of mAb-based immunoconjugates are discussed, followed by the critical criteria in their selections for designing and developing efficacious tumour-targeting chemotherapeutic agents.

2. Composition of immunoconjugates

The mAb-based immunoconjugate consists of three parts (Figure 1); the mAb, the warhead and the linker. The mAb is the vehicle that carries the conjugate to the target site, based on the recognition of specific antigens that are overexpressed on the surface of the tumour cells. On binding, a mAb–antigen interaction triggers internalisation via endocytosis. The warhead is an agent with strong cytotoxicity to kill the tumour cell. The role of the linker is to connect the mAb with the warhead(s) to form an inactive prodrug whilst in systemic circulation. Early works on immunoconjugates involved a direct connection of a warhead and mAb by an amide bond. This method suffered from an insufficient release of the active drug (i.e., the warhead). These failures led to the development of cleavable linkers.

The conjugate must exhibit a good pharmacokinetic profile, optimal biodistribution and wide therapeutic window. Consequently, there are various requirements for the selection of a suitable mAb, cytotoxic agent and linker to construct an efficacious immunoconjugate.

Conjugates are generally prepared by employing suitable chemical methods to covalently bind a mAb and cytotoxic agent through a linker. Alternatively, gene- and protein-engineering can be used for the preparation of immunotoxins as fusion proteins by expression in bacteria, although the

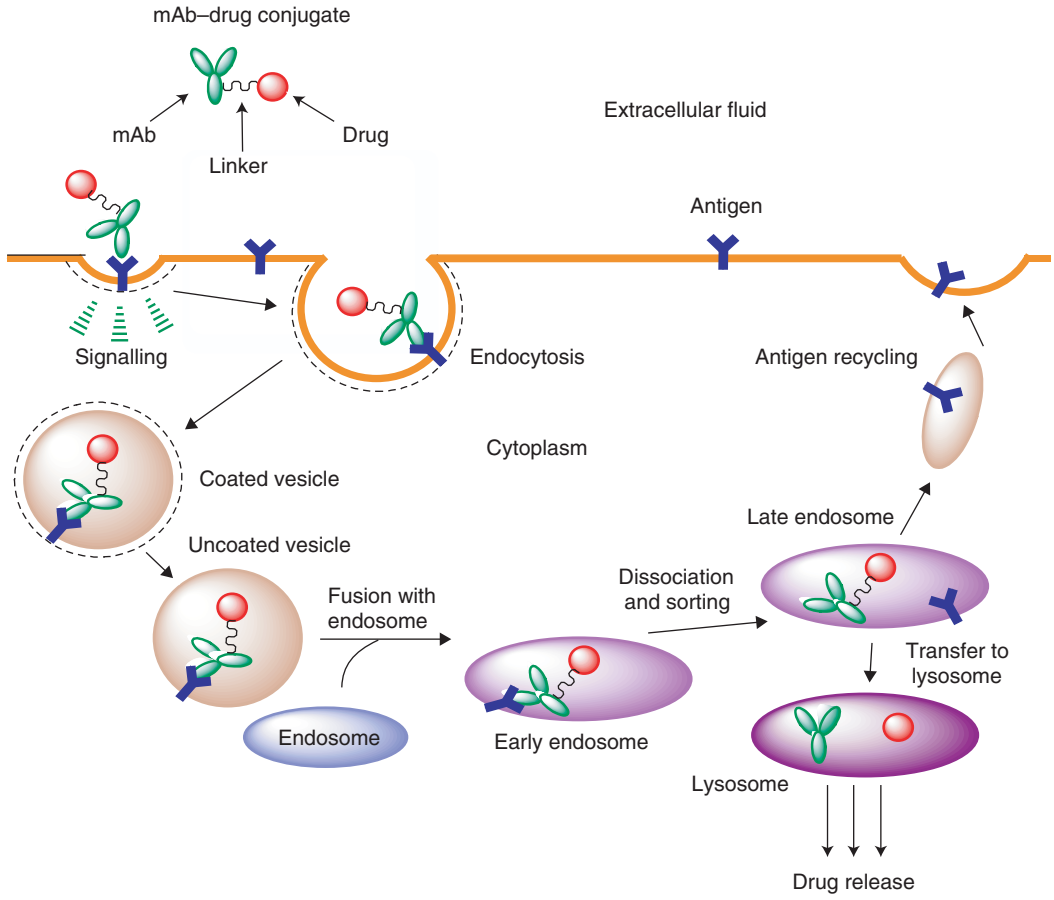


Figure 2. Endocytosis
mAb: Monoclonal antibody.

scope is still rather limited [14,15]. Fusion proteins are composed of a ligand derived from a mAb fused to a truncated plant or bacterial toxin.

On binding to the antigen on the tumour surface, a mAb–cytotoxic agent conjugate is internalised into the tumour cell mostly via receptor-mediated endocytosis. This process involves several steps as shown in Figure 2. In the first step, the complementarity-determining regions (CDRs) on the mAb recognise and bind to antigens on the tumour cell surface. Typically, the resulting complex is internalised to form a vesicle coated by clathrin that is subsequently removed by depolymerisation. The resulting uncoated vesicle fuses to an endosome in the cell to form a new endosome ('early endosome') wherein the pH value drops gradually from 7 to 5. This change in pH causes the dissociation of the immunoconjugate from its receptor, which takes place in the 'late endosome'. Certain types of antigens can be recycled to the membrane, whereas mAb–cytotoxic agent conjugates remain in the late endosome, which is then transferred to a lysosome. Depending on the linker employed, the cytotoxic agent is released from the conjugate by various mechanisms, such as proteolysis, acid-catalysed hydrolysis or disulfide exchange.

Because only a limited number of antigens are present on the cancer cell surface, the attachment of several molecules of a cytotoxic agent on a single mAb is preferred. One of the ways to increase the loading of cytotoxic molecules is to design a multivalent linker that can carry multiple warheads [16,17]; however, it has been shown that excessive loading causes enhanced immunogenicity and nonspecific toxicity in mice *in vivo* [18]. In addition, a high loading of hydrophobic cytotoxic molecules impairs mAb binding affinity due to aggregation and denaturation [4]. Thus, the optimal loading of cytotoxic molecules per mAb is a key design parameter for the construction of efficacious immunoconjugates.

3. Monoclonal antibodies

The discovery of antigens that are specifically overexpressed on the surface of cancer cells suggests a possible use of antigen-specific mAbs to selectively 'mark' tumour cells. Thus, cancer cells could be distinguished from normal cells. A mAb has a

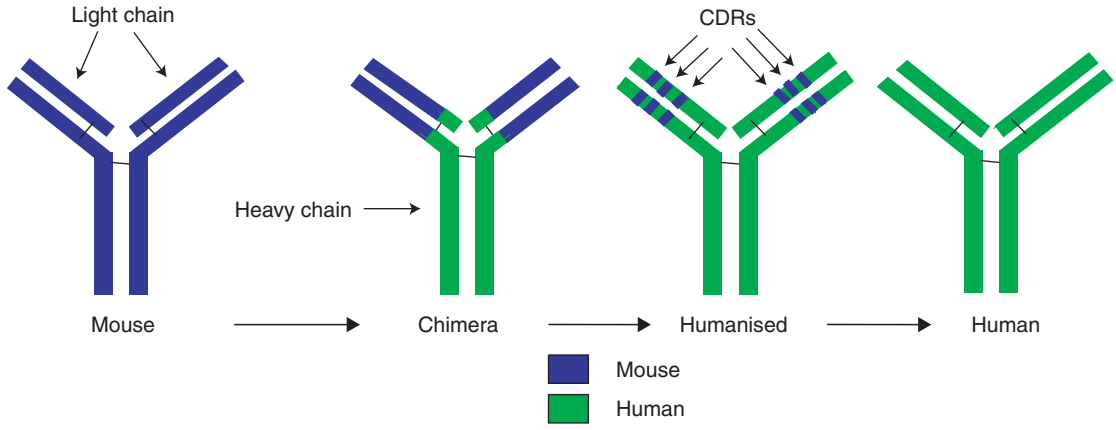


Figure 3. Evolution in the monoclonal antibody technology (schematic representation of the development of humanised monoclonal antibodies) [19,301]. Adapted from [301] therapeutic antibody, page 7, with permission from Dr Masayuki Tsuchiya.

characteristic general structure, consisting of two identical light chains and two identical heavy chains, which are linked by disulfide bonds. The mAb could be cleaved, through limited proteolysis with papain into three ~ 50-kDa fragments; two identical Fab (antigen binding) fragments and one Fc (crystallisable) fragment. In 1975, Kohler and Milstein [2] described their pioneering concept and work on mAbs by hybridoma technology, which was rewarded by the Nobel Prize in Physiology or Medicine in 1984. However, the murine antibody derived from the hybridoma technology has limited therapeutic utility due to the human antimouse antibody (HAMA) response, which results in the rapid clearance of the immunoconjugate from the bloodstream. Accordingly, recombinant DNA technology was developed, which produced generations of chimeric and humanised mAbs with decreased immunogenicity (Figure 3) [19]. Currently, the most widely used mAbs are the humanised antibodies, which are created by grafting the CDR from a mouse mAb into a human IgG. The binding affinity of the humanised mAb can be fully preserved compared with the original murine mAb [20].

MAbs can play two kinds of roles in cancer chemotherapy (i.e., as a drug as it is or it can serve as a tumour-targeting molecule). Although several antitumour antibodies, such as rituximab, trastuzumab, alemtuzumab and bevacizumab, have been approved by the FDA for cancer therapy, their antitumour activities are not necessarily sufficient; for example, in the clinical trials of bevacizumab (Avastin™), which was approved by the FDA for the treatment of metastatic colorectal cancer in February 2004, the overall response rate was ~ 40% and the patients' lives were prolonged by 4.7 months in average [302]. In the next step, the development of mAb–cytotoxic drug immunoconjugates is a logical strategy to improve the efficacy of both the mAb and cytotoxic agent.

There are a variety of functional groups available for the attachment of the cytotoxic agent to a mAb. One of the most reactive groups in a mAb is the amine moiety in the side chain of a lysine residue. A molecule of mAb contains ~ 90 lysine residues [21]. These amine moieties are good nucleophiles that react easily and cleanly with activated carboxylic acids to form stable amide bonds. Another useful functional group for the construction of an immunoconjugate is the thiol moiety of a cysteine residue. Because the thiol functionality in proteins is sensitive to oxygen, cysteines are often oxidised to disulfide-containing dimers, cystines. The reducing reagent dithiothreitol (DTT) is commonly used to generate the free sulfhydryl groups in a mAb for linker attachment via a disulfide or thioether bond. Vicinal diols in a carbohydrate moiety can be readily oxidised by sodium periodate to generate two corresponding aldehyde groups, which can react with functional groups, such as amine, hydrazine or hydrazide, in a modified molecule of cytotoxic agent [22]. As the carbohydrate moiety is localised in the Fc region of the mAb, it is believed that the attachment of a cytotoxic agent would not affect the antigen-binding affinity of the mAb [23]. Generally, the loading number of cytotoxic molecules on a mAb is 3 – 8 [23].

Current mAb-based chemotherapy appears to be limited by the physical properties of mAb molecules with a molecular weight of ~ 150 kDa. Some mAb–cytotoxic agent conjugates did not show the anticipated activities in the treatment of solid tumours, because of rather poor tumour penetration and an insufficient pharmacokinetic profile [24,25]. To overcome this limitation, a mAb technology was developed to generate a Fab, $F(ab')_2$ (two covalently linked Fab′) or scFv (single-chain variable) fragment as a tumour-targeting protein [1,26-28]. The biological evaluation of such truncated immunoconjugates is currently under active investigation [1,26-28].

4. Cytotoxic agents

The selection of an appropriate cytotoxic agent for receptor-mediated drug delivery must meet several criteria. The first

criterion is high potency. As only a limited amount of cytotoxic molecules can be loaded onto a mAb without affecting its binding affinity to antigens, highly potent cytotoxic agents are preferred. It has been calculated that the maximum concentration of the drug delivered to a cancer cell through receptor-mediated endocytosis would probably not exceed 10^{-7} M; therefore, the toxicity of the warhead should be in the subnanomolar range to be effective in humans [4,29]. Various alkylating agents or antimetabolites (e.g., triazenes, melphalan, chlorambucil and methotrexate [30]) require relatively high concentrations to be effective, which makes them unsuitable for use in mAb–cytotoxic agent conjugates. The second criterion is the stability of the cytotoxic agent not only in the bloodstream but, more importantly, in the proteolytic and acidic conditions of lysosomes where the cytotoxic agent is released. The third criterion is the lack of immunogenicity. Cytotoxic agents of a large molecular size, especially bacteria and plant toxins, can be potentially destroyed by the host immune system. The fourth criterion is the presence of proper functional groups, such as hydroxyl, amino, sulfhydryl, carbonyl and carboxyl groups, which can be easily modified to attach to a linker. The fifth criterion is insensitivity towards multi-drug resistance (MDR) or bearing MDR-reversal activity. The cytotoxic agent or its active metabolite should not leak extensively from the cell by efflux mechanism or diffusion.

4.1 Non-protein cytotoxic agents

Neocarzinostatin (Figure 4) is a protein–chromophore complex obtained from the culture broth of *Streptomyces carzinostaticus*. Its original structure, reported in 1966 [31,32], was revised several times until confirmation in 1993 [33]. Neocarzinostatin is composed of a labile chromophore and stabilising protein (113 amino acids, 11 kDa). The active chromophore component contains an enediyne unit capable of efficient DNA cleavage [34,35].

Calicheamicin (Figure 4), containing an enediyne with an unusual trisulfide moiety and an iodine atom, is produced by *Micromonospora echinospora calichensis*. Calicheamicin binds to a minor groove of DNA and produces sequence-specific DNA breaks [36]. Calicheamicin shows remarkable potency against various tumours and is ~ 4000-fold more active than doxorubicin (adriamycin), with an optimal dose of 0.5 – 1.5 µg/kg [37,38]. The single-agent chemotherapy has been limited because of delayed toxicities, which lead to a very narrow therapeutic window. The unique mechanism of action and extreme potency make calicheamicin a good candidate for immunoconjugates.

Doxorubicin and daunorubicin (Figure 4), isolated from cultures of *Streptomyces peucetius* in 1963 [39] and 1968 [40], respectively, belong to the anthracyclin group of cytotoxic agents. These two drugs gained immediate attention as potent antitumour antibiotics due to their broad-spectrum activity. Numerous immunoconjugates of doxorubicin with mAbs have been investigated [17,41-47].

Maytansine (Figure 4) was isolated from the *Maytenus ovatus* plant, and its structure was elucidated in 1972 [48]. It is a chlorine-containing macrolactam with an epoxide ring and the initial *in vitro* studies showed median effective values (ED_{50}) in the range of 0.6 – 2 nM [49]. Maytansine is a powerful inhibitor of microtubule assembly [50]. The synthetically modified maytansine analogue, methyldithio-maytansinoid (DM1), shows 100- to 1000-fold higher cytotoxicity than doxorubicin, methotrexate and vinca alkaloids, with a median inhibitory concentration (IC_{50}) in the picomolar level. DM1 has been investigated for mAb-based tumour-targeting therapy by Immunogen [9].

Mitomycins A, B and C (Figure 4) were isolated from the soil bacteria *Streptomyces verticillatus* [51]. Their structures were determined in 1962 [52]. The mitomycins form a specific class of antitumour antibiotics and act as DNA alkylating agents [53,54]. Mitomycin C was investigated for the mAb-based tumour-targeting chemotherapy against human gastric cancer and biliary tract carcinoma xenografts in mice [55-57].

Paclitaxel (Taxol®; Figure 4) is an antileukaemic and antitumour agent originally isolated from the bark of the Pacific yew tree, *Taxus brevifolia* [58]. The mechanism of action of paclitaxel involves an acceleration of tubulin polymerisation and stabilisation of the resultant microtubules [59,60].

The application of paclitaxel in immunoconjugates has not shown significant efficacy *in vivo* [61,62]. The observed inefficacy of mAb–paclitaxel conjugates can be ascribed to insufficient potency, insufficient intracellular release of the drug or unfavourable effects on the mAb function due to the high hydrophobicity of the drug. In addition, paclitaxel is ineffective against drug-resistant cancer cells expressing MDR phenotypes. In sharp contrast, most of the second-generation taxoids developed by Ojima *et al.* [63-67], for example SB-T-1213 (Figure 4), exhibit one order of magnitude higher potency than that of paclitaxel against drug-sensitive cancer cell lines. They also showed two to three orders of magnitude higher potency than that of paclitaxel against MDR-expressing cell lines. These properties make them highly promising candidates as warheads for efficacious mAb–cytotoxic agent conjugates. Accordingly, the conjugates of the second-generation taxoids with mAbs have been studied. These immunoconjugates show high potency and exceptional tumour-targeting specificity [68-70].

Auristatins are synthetic analogues of dolastatin 10, a pentapeptide isolated from the sea hare *Dolabella auricularia* [71-72]. The antitumour activity of extracts from *D. auricularia* was discovered in 1972 and, after structure elucidation of dolastatin 10, the activity was demonstrated in various tumour models [73-74]. This class of compounds exerts potent antitumour activities through the inhibition of tubulin polymerisation. Auristatins are 100- to 1000-fold more potent than doxorubicin and can be prepared in large quantities. Auristatin E exhibits an average IC_{50} value of 3.2 nM against a diverse panel of human tumour cell lines, including

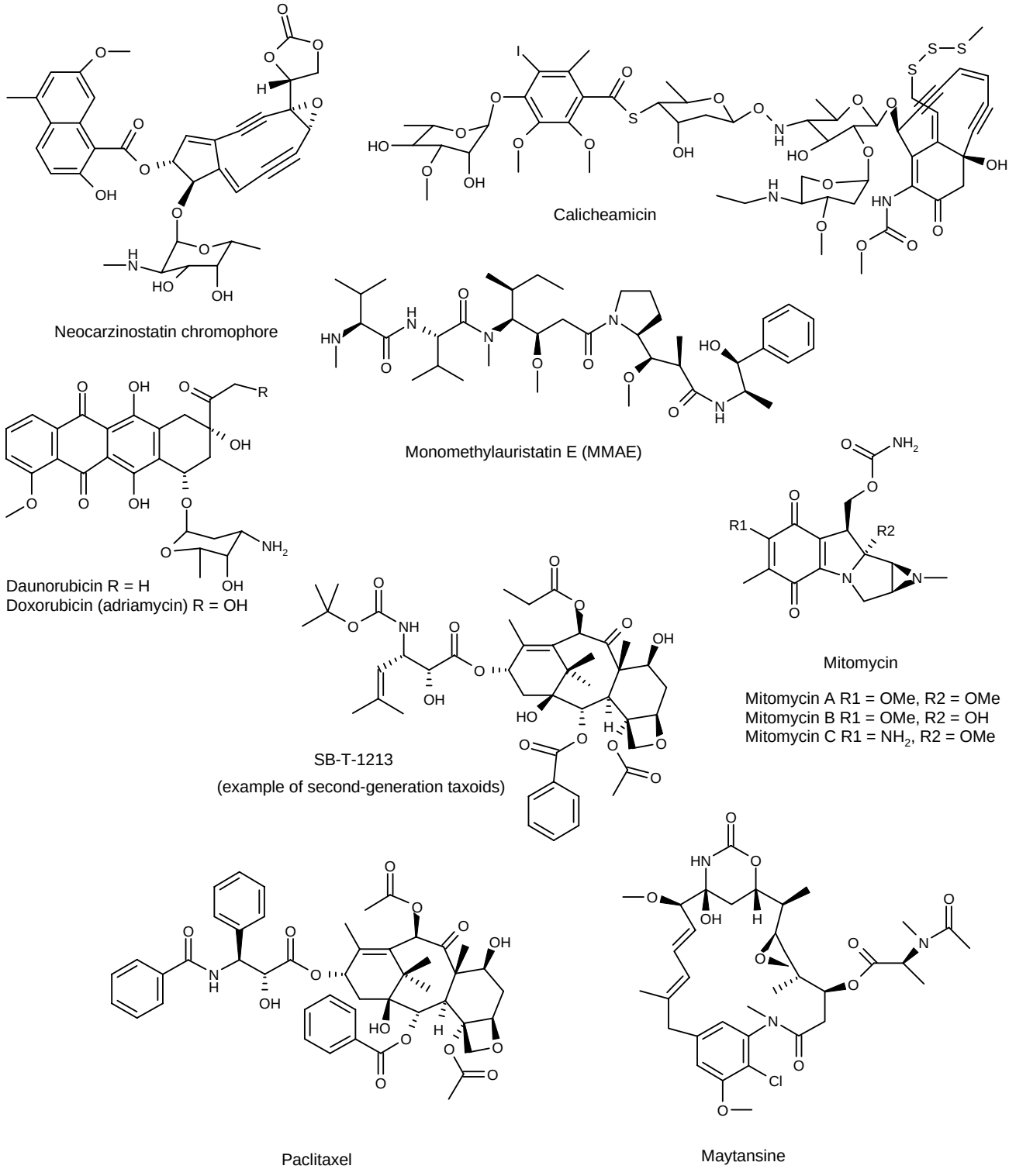


Figure 4. Cytotoxic agents used in monoclonal antibody–cytotoxic agent conjugates.

haematological malignancies, melanoma, carcinomas of the lung, stomach, prostate, ovaries, pancreas, breast, colon and kidneys [75]. Another potent dolastatin 10 analogue, monomethylauristatin E (MMAE; Figure 4), was linked to a mAb and the resulting immunoconjugate exhibits promising antitumour effects against a Karpas 299 lymphoma *in vitro* and *in vivo* (see Section 5.2) [75].

4.2 Bacteria protein toxins

Diphtheria toxin is an enzyme secreted by the bacteria *Corynephage beta*. The active form consists of two polypeptide chains, A (21 kDa) and B (37 kDa), with a total of 560 amino acid residues [76]. The mode of action involves the attachment of an ADP-ribose group to the elongation factor 2, thereby halting cellular protein synthesis. As the inhibition of the protein synthesis is a catalytic process, only a minute amount of the toxin is necessary [77,78]. Diphtheria toxin-containing conjugates have been developed through fusion protein technology [79-81]; for example, the immunotoxin-containing diphtheria toxin and human IL-2 has been approved for the treatment of cutaneous T-cell lymphoma. This immunotoxin is also effective in patients who developed resistance during previous chemotherapy [79].

Pseudomonas exotoxin A of *Pseudomonas aeruginosa* is a virulence factor secreted in response to a scarcity of iron. The 66-kDa protein has 613 amino acid residues and 4 disulfide linkages. It is highly toxic to eukaryotic cells and causes the arrest of protein synthesis through the same mechanism as the diphtheria toxin. The median lethal dose for a mouse is 5 µg/kg [82, 83]. Because activation of the toxin *in vivo* requires several steps, active truncated (37 kDa) or mutated analogues are usually employed [84]. Pseudomonas exotoxin A is the most frequently used protein toxin to form immunotoxins by fusion protein technology [85-87]; for example, a conjugate of truncated pseudomonas exotoxin A with single-chain fragment of mAb 8H9 (62 kDa) was biosynthesised following its expression in genetically engineered *Escherichia coli* [86]. This conjugate shows cytotoxicity ranging from 5 to 90 ng/ml against nine different cancer cell lines (breast cancer, osteosarcoma and neuroblastoma). The antitumour activity was also evaluated *in vivo* against breast cancer and osteosarcoma xenografts in severe combined immunodeficient (SCID) mice. The immunotoxin also exhibited a specific dose-dependent antitumour activity at 0.075 and 0.15 mg/kg. Toxicological studies on cynomolgus monkeys show that this immunotoxin was well tolerated.

4.3 Plant protein toxins

Ricin is an abundant 66-kDa protein toxin from the castor bean plant seeds *Ricinus communis* and it is one of the most poisonous plant toxins. It is composed of two peptide chains connected by a disulfide bond. The A chain (32 kDa) is an enzyme that catalyses *N*-glycosidic cleavage, and the B chain (34 kDa) is a galactose-binding lectin. Ricin inhibits protein synthesis by specifically and irreversibly inactivating eukaryotic ribosomes [88-89]; for example, the A chain has been attached to a rat mAb that can recognise mouse and human prostate-specific membrane antigen (PSMA). This transmembrane protein is largely restricted to prostatic epithelial cells in humans and is strongly upregulated on prostatic carcinoma cells. The ricin–immunoconjugate strongly inhibits the growth of PSMA-positive LnCaP cells with an IC_{50} of 60 pM. The ricin–immunoconjugates were administered to nude mice bearing human prostatic tumour xenografts in one 50 µg intraperitoneal dose with a repetition after 7 days. After 2 weeks, the tumour growth was retarded by eightfold when compared with non-targeted control without causing apparent systemic toxicty [90].

Abrin is a toxic protein obtained from the seeds of *Abrus precatorius* (jequirity bean), which is similar in structure and properties to ricin [90-92]. Abrin is highly toxic, with an estimated human fatal dose of 0.1 – 1 µg/kg [93].

Saporin, a monomeric protein extracted from the seeds of *Saponaria officinalis*, is an enzyme capable of specific depurination of the eukaryotic ribosomes. A saporin immunotoxin targeting epidermal growth factor receptor (EGFR) significantly inhibited tumour cell growth and protein synthesis as well as inducing substantial apoptosis in rhabdomyosarcoma cells [94]. However, the application of saporin in a mAb-based immunoconjugate has only had limited success due to its low efficacy *in vivo* [94].

It should be noted that the major impediments with immunotoxins (of both bacterial and plant origin) in their clinical applications are immunogenicity [95], causing reduced therapeutic effect by fast clearance of the immunotoxins from the crculation and vascular leak syndrome [96,97], characterised by an increase in vascular permeability resulting in interstitial oedema and organ failure.

5. Linkers

Although the linker represents the smallest part of a mAb–cytotoxic agent conjugate, it has a critical significance in the efficacy of the immunoconjugate. Selection of a suitable linker should meet a few stringent criteria. First, the linker must be stable in circulation but efficiently cleaved inside cancer cells; however, a linker that is too stable within the tumour prevents the release of the cytotoxic agent from the conjugate, which would result in substantially decreased or insufficient cytotoxicity. Second, the attachment of cytotoxic molecule(s) should not alter the ability of mAb to recognise its specific antigen or increase its immunogenicity; thus, there is a limitation in the number of cytotoxic molecules that can be attached to a mAb. Third, a linker and a cytotoxic agent should be attached to a mAb under mild conditions to avoid denaturation of the mAb. Fourth, the linkers should be well exposed so that they can be readily cleaved in cancer cells.

The most frequently used linkers can be categorised into four classes in accordance with the modes of cleavage: acid labile; proteolytic; disulfide exchange and hydrolytic linkers (Figure 5). Selection of an appropriate linker depends on the

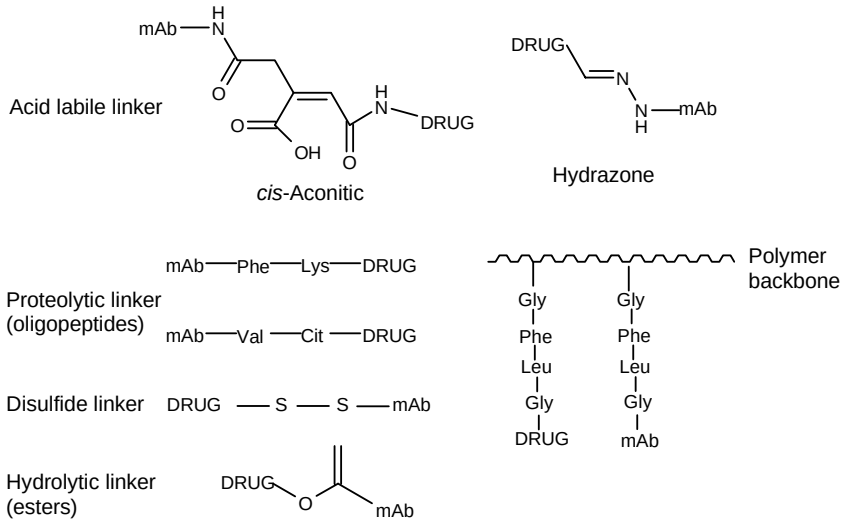


Figure 5. Linker structures.

type of cancer and the required cytotoxic agent. None of the linkers is universal and each of them has advantages and disadvantages. In the following sections, specific examples are described and the characteristics and applicability of various linkers are discussed.

5.1 Acid-labile linkers

The acid-labile linkers take advantage of the acidic (~ pH 5) conditions in the endosomes and lysosomes to release the parent cytotoxic agent via non-enzymatic hydrolysis. *cis*-Aconitic and hydrazone linkers have been extensively studied.

5.1.1 *cis-Aconitic linker*

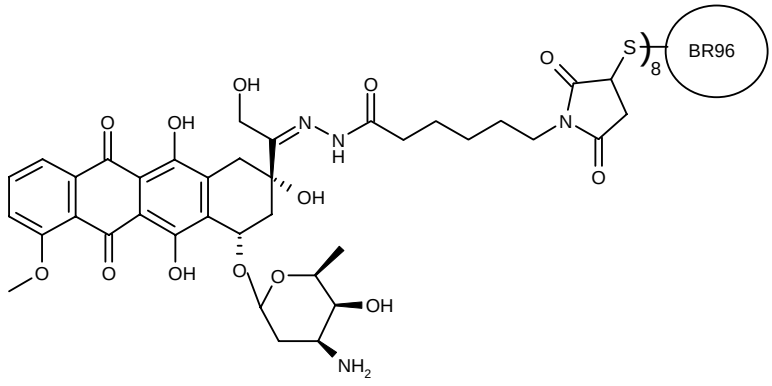
cis-Aconitic acid is one of the first acid-labile linkers used in immunoconjugates. The very first application of this linker was reported by Shen and Ryser [98] to attach daunorubicin to poly-D-lysine. Later, this linker was also used to conjugate daunorubicin to the anti-T cell mAb by Yang and Reisfeld [99] as well as Dillman *et al*. [100].

5.1.2 *Hydrazone linker*

Hydrazone-containing linkers are the most extensively used acid-labile linkers. The cytotoxic molecule must have a suitable ketone or aldehyde moiety in order to form a hydrazone. Alternatively, the carbonyl groups can be on the mAb as well. Vinblastine [101], doxorubicin [102,103], daunorubicin [201], chlorambucil [201] and calicheamicin [8] have been used as cytotoxic agents in tumour-targeting immunoconjugates containing a hydrazone linker.

The hydrazone linker was used for the first time in 1990 to attach doxorubicin to a mAb [104]. This type of conjugates exhibited a higher release rate under slightly acidic conditions than neutral conditions; however, premature cleavage of the hydrazone linker at physiological pH was also observed [102], which indicates some instability of this linker during circulation. Doxorubicin was also attached via a hydrazone linker to a chimeric mAb BR96 that can recognise Lewis Y antigens abundantly expressed on various human carcinomas, such as lung, breast and colon (Figure 6). The mAb–cytotoxic agent conjugate containing eight molecules of doxorubicin was rapidly internalised on binding to the antigen [4]. Complete regressions of human breast, lung and colon carcinomas were observed for xenografts in animal models [103,105]; however, the Phase II clinical trials show that the single-agent treatment was not efficacious enough to cause complete responses in patients with large solid tumours. Follow-up studies in lung, colon and breast tumour xenograft models demonstrated that a combination therapy using BR96–doxorubicin with paclitaxel or docetaxel results in significant synergistic antitumour activity [106].

CDR-grafted humanised mAb P67.6 targeting CD33 in leukaemia cells was linked to calicheamicin via a hydrazone linker (Figure 7). The immunoconjugate containing two or three molecules of calicheamicin has a rather sophisticated linker system. The lysine residue in mAb was coupled to 4-(4-acetylphenoxy)butanoic acid via a stable amide bond. The carbonyl group of the acetophenone moiety was then linked to calicheamicin via an alkanoylhydrazone linker serially connected to a disulfide moiety. It has been shown that the hydrazone linker is the actual cleavage site [107]. Cleavage by disulfide exchange was insufficient. The *in vitro* assay shows that the conjugate was 2000-fold more potent than the parent cytotoxic agent. This remarkable activity was also confirmed *in vivo*. After successful human clinical trials, the immunoconjugate (i.e., gemtuzumab–ozogamicin, Mylotarg) was approved by the FDA for the treatment of acute myelogenous leukaemia in



.**Figure 6. BR96–doxorubicin conjugate** [103,105]**.**

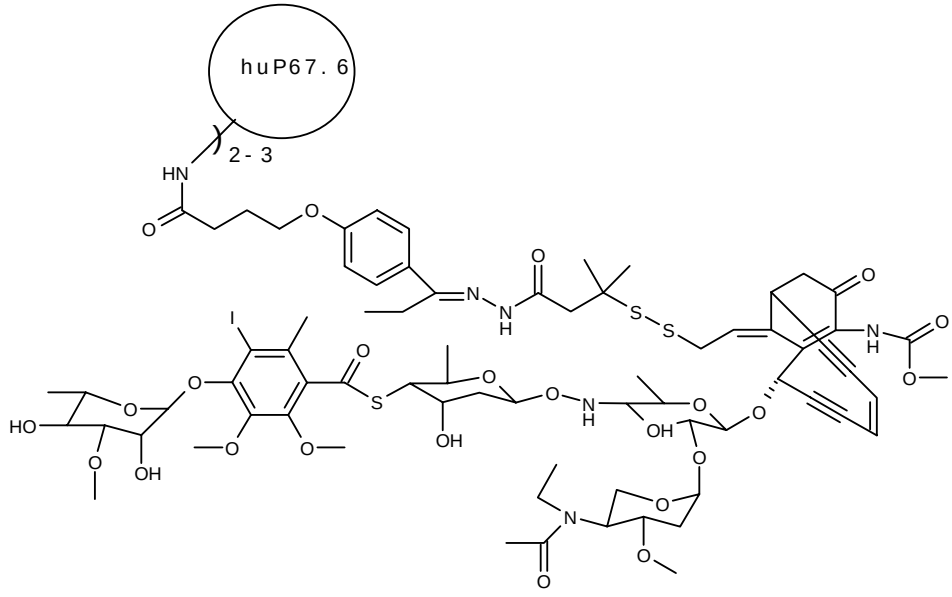


Figure 7. huP67.6–calicheamicin (Mylotarg, gemtuzumab–ozogamicin) [8,107]**.**

2000, thus becoming the very first mAb-conjugated anticancer drug on the market [8].

5.2 Peptide linkers

Conjugates delivered to lysosomes in a cancer cell via internalisation are exposed to many proteases, mostly belonging to the cathepsin family and a number of exopeptidases. A number of short peptide sequences were investigated for their use as a linker. The peptide linker should have two to five amino acid residues with at least two hydrophobic, preferably aromatic, residues [108]. Examples of peptide linkers include the tetrapeptide Gly-Phe-Leu-Gly [109] as well as dipeptides, such as Phe-Lys and Val-citrulline (Cit) [110].

The dipeptide linkers Phe-Lys and Val-Cit have been used for the immunoconjugates of doxorubicin with BR96. In addition, a 'self-immolative' *p*-aminobenzyloxycarbonyl (PABC) spacer was inserted between the dipeptide and cytotoxic agents. The self-immolation is triggered by a proteolytic cleavage of the linker by cathepsin B in the lysosomal compartments of tumour cells [111]. The half-lifes of the Phe-Lys-PABC and Val-Cit-PABC linkers were 8 and 240 min, respectively. No cleavage was detected when incubated in fresh human plasma over 7 h. The *in vitro* experiment showed potent antigen-specific cytotoxicity of both immunoconjugates with IC_{50} values of 0.15 and 0.4 µM, respectively.

The linker Val-Cit-PABC and BR96 were also employed for the formation of a conjugate with MMAE by Seattle Genetics (Figure 8) [75]. The *in vitro* cytotoxicity assay of this conjugate against the Karpas 299 cell line yielded an IC_{50} value of 4.5 ng/ml and the specificity ratio (the IC_{50} ratio of

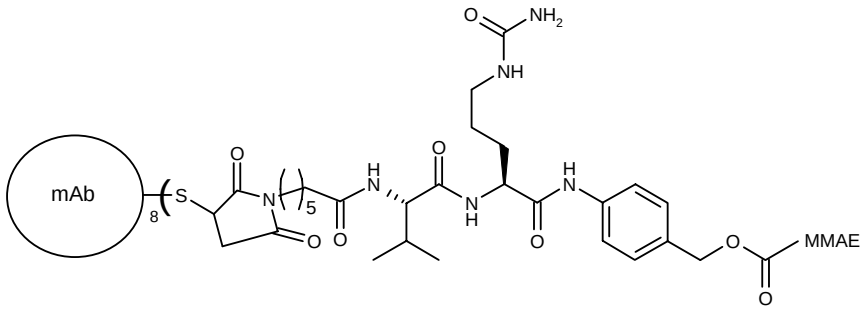


Figure 8. MAb–MMAE conjugate [75].
MMAE: Monomethylauristatin E.

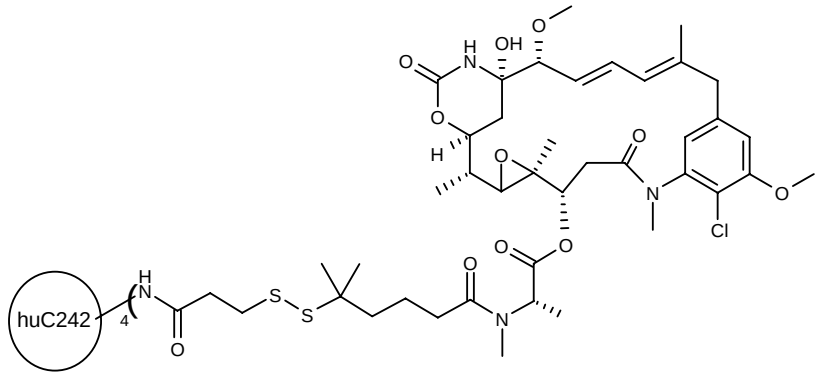


Figure 9. huC242–DM1 conjugate [9,107].

nonbinding conjugates to the binding control) of > 500. The *in vivo* antitumour activity assay against Karpas 299 lymphoma in SCID mice showed that the effective dose was 60-times lower than the maximum-tolerated dose (MTD). Thus, MMAE 35 µg component/kg/injection achieved 100% tumour cure in human Karpas 299 anaplastic large cell lymphoma.

5.3 Disulfide linker

The disulfide linker is cleaved inside tumour cells through disulfide exchange with an intracellular thiol such as glutathione. The concentration of glutathione inside the cell is higher than that in the serum [112-115]. Moreover, the concentration is also much higher in the tumour cell compared with the normal cell [116]. In order to stabilise a disulfide linker against premature cleavage in circulation, sterically hindered disulfides are commonly used in the construction of prodrugs. The following examples provide a strong indication that disulfide linker-containing immunoconjugates have superior efficacy to other linkers against several tumour xenografts, including colorectal, pancreatic, gastric, small-cell lung, non-small-cell lung, prostate and breast cancers in preclinical models [9,10].

A specially designed maytansine derivative DM1 bearing a methyldisulfanyl (MDS) group was attached to a humanised mAb huC242, which has high binding affinity to the CanAg antigen expressed on most pancreatic, biliary and colorectal cancer cell membranes (Figure 9). The immunoconjugate bearing a disulfide linker showed remarkable potency and selectivity *in vitro* and *in vivo*. The mAb–cytotoxic agent conjugate cured all mice bearing COLO 205 human colon tumour xenografts at a much lower dose than the MTD. Moreover, treatment with the immunoconjugate also produced complete regressions in animals bearing COLO 205 tumour xenografts of a large size (260 – 500 mm^3) [9]. A Phase I clinical trial on 37 patients with CanAg-expressing solid malignancies observed some responses with the terminal elimination half-life of 41 h (± 16). A small amount (< 1%) of prematurely cleaved maytansinoid DM1 was also detected in the blood [117].

Besides huC242, several other mAbs were also linked to DM1 using a similar strategy to form immunoconjugates, including huN901–DM1, MLN2704–DM1, trastuzumab–DM1 and CD44v6–DM1 (bivatuzumab–mertansine). These prodrugs are currently in Phase I or II clinical studies [10,118,119].

A disulfide-containing CC-1065 analogue derivative was conjugated to anti-B4 and N901 targeting CD19 and CD56,

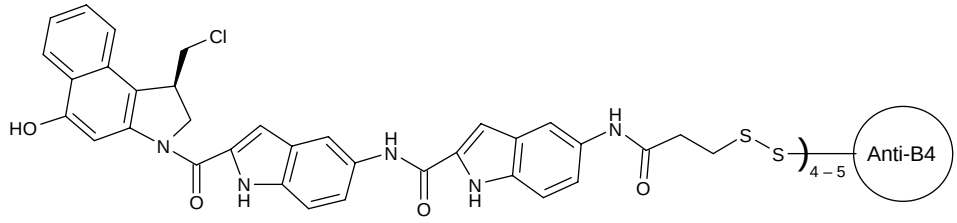


Figure 10. Anti-B4–CC-1065 analogue conjugate [4,120]**.**

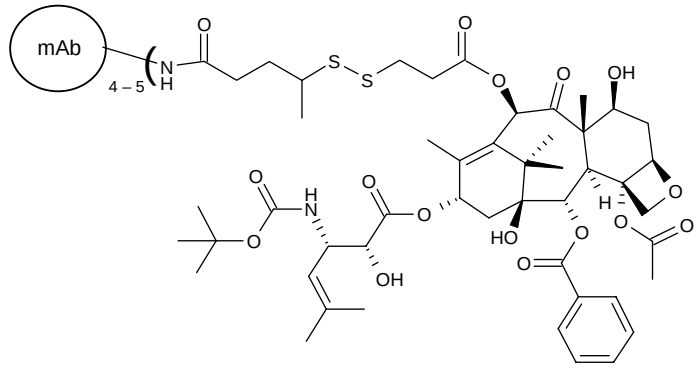
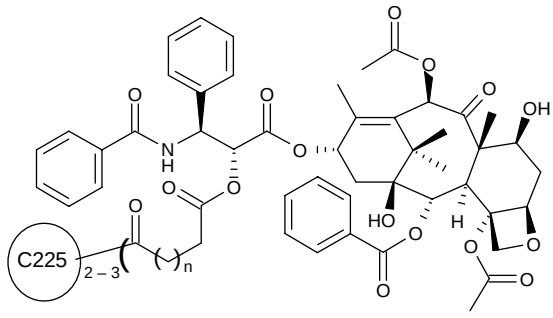


Figure 11. mAb–SB-T-12136 conjugate [68]**.**
mAb: Monoclonal antibody.

respectively (Figure 10). Each immunoconjugate contains three or four disulfide-linked molecules of CC-1065 analogue. The conjugate antiB4–CC-1065 analogue is cancer cell specific and highly cytotoxic with the IC_{50} value of 0.06 nM. Comparison of the cytotoxicity of anti-B4–CC-1065 analogue against non-targeted and targeted cells demonstrated > 700-fold higher potency against the targeted cells [4]. The disulfide linkage in this system appears to be very stable because the exposure of the conjugate to antigen-negative cells for 21 days did not show any cytotoxicity. The *in vivo* assay was performed against an aggressive metastatic human B cell lymphoma model in SCID mice [120]. Treatment with anti-B4–CC-1065 analogue induced 265% of a median increase in survival time compared with 22 – 91% for the anticancer drug alone.

In a manner similar to gemtuzumab–ozogamicin, calicheamicin was also conjugated to an anti-MUC1 mAb through a bifunctional linker containing both hydrazone and disulfide moieties [121]. The loading of the cytotoxic agent was two or three molecules per mAb. Further study without the hydrolytic linker indicate that the disulfide bond was the only cleavable site. The mAb–cytotoxic agent conjugate with the disulfide linker exhibited equivalent or superior activity to the conjugate with the bifunctional linker, especially against drug-resistant cell lines [107]. A Phase I clinical trial was performed on female patients with epithelial ovarian cancer [122]. Some therapeutic responses were obtained but relatively severe side effects were also observed due to the expression of the target antigens on normal tissues [115].

Ojima *et al.* [68] developed mAb–taxoid conjugates with disulfide linkers. Second-generation taxoid SB-T-1213 was chosen to form mAb–cytotoxic agent conjugates (Figure 11). Structure–activity relationship studies clearly demonstrate that cytotoxicity is retained when a MDS-propanoyl group was attached to the C-10 position of the taxoid. The resulting 10-MDS-alkanoyl analogue of SB-T-1213, SB-T-12136, was linked to the mAbs KS-61, KS-77 and KS-78 that specifically bind to human EGFR, which is overexpressed in several human squamous cancers such as head, neck, lung and breast cancers. These immunoconjugates exhibited specific cytotoxicity (IC_{50} ~ 1.5 nM) against human squamous cancer A-431 cancer cells expressing EGFR. In vivo antitumour activities of two conjugates, KS61–SB-T-12136 and KS77–SB-T-12136, were evaluated against A-431 xenografts in SCID mice. Both conjugates showed remarkable antitumour activity, resulting in a complete inhibition of tumour growth of all the treated animals for the duration of the experiment. Necropsy on day 75, followed by a histopathological examination, showed residual calcified material at the tumour site but no evidence of tumour cells. Free taxoid SB-T-12136 at the same dose showed no therapeutic effect. Moreover, the immunoconjugates were well-tolerated by the mice as demonstrated by the absence of any weight loss.



n = 1 or 2

Figure 12. C225–paclitaxel conjugate [123,124]**.**

5.4 Ester linker

Ester linker is cleaved due to pH sensitivity or by lipase-catalysed hydrolysis *in vivo*. The high lability of this linker limits its use in prodrug construction without proper modification; thus, in order to circumvent this obstacle, sterically hindered secondary alcohols are used.

Safavy *et al.* [123,124] reported the synthesis and biological evaluation of the paclitaxel–C225 conjugate for tumour-targeting therapy (Figure 12). The C225 mAb specifically recognises EGFR. The conjugate was formed by connecting the mAb to the C2′-hydroxyl group of paclitaxel through an ester bond using succinic acid as a spacer. *In vitro* treatment with this conjugate induced more apoptosis than the free drug alone. However, an *in vivo* study showed that there is no difference between the conjugate and C225 in inhibiting the tumour growth. It is very likely that this is due to the instability of the ester linker. The same research group developed a [^{125}I]labelled 2′-OH-paclitaxel–C225 conjugate. A biodistribution and *in vitro* kinetic study confirmed that the conjugate is not stable under physiological conditions with a half-life of ~ 2 h. To improve the systemic stability of the conjugate, the succinic acid moiety in the conjugate was replaced by glutaric acid [124]. The new prodrug showed much better stability and its *in vivo* study also indicated better antitumour activity [124].

6. Polymer-based immunoconjugates

The use of polymer-based immunoconjugates has been proven to be a promising approach for the treatment of cancer. It is expected that the immunoconjugates would benefit from the introduction of polymers in two aspects. First, the enhanced permeability and retention (EPR) effect would lead to prolonged release of a cytotoxic agent due to the increased blood clearance time and reduced filtration of the conjugates in the kidney [125]. Secondly, the potency of immunoconjugates could be improved by increasing the drug loading per mAb without diminishing the mAb's binding affinity.

To date, the most successful approach reported is based on the use of biodegradable *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer. A HPMA-based immunoconjugate bearing covalently linked doxorubicin through a biodegradable Gly-Phe-Leu-Gly tetrapeptide has been successfully developed and used for the treatment of five patients with generalised breast carcinoma in the Czech Republic [126]. Ulbrich *et al.* [127,128] synthesised HPMA-based immunoconjugates with a classical and star structure (Figures 13 and 14). An *in vivo* animal model study showed that the HPMA conjugate with the star structure exhibited significantly higher antitumour efficacy than that with the classic structure. This is probably due to the fact that doxorubicin in the star structure is much better exposed compared with that in the classical structure, which results in more efficient intracellular release of the drug.

Janda *et al.* [26] reported a practical approach to design immunoconjugates containing dendrimers. The dendritic molecules with a polyamidoether backbone were used as multivalent drug carriers. Up to nine chlorambucil molecules were linked to the dendritic backbone. This complex is ready to attach to a scFv fragment and the resulting conjugate is expected to show better tumour penetration than the conjugate containing an intact mAb. The dendritic strategy could effectively increase the drug loading and is suitable for studying the relationship between the drug/mAb ratio and the activity of the immunoconjugates. However, the efficacy of this approach must wait for the results of antitumour activity evaluation *in vivo*.

7. Expert opinion and conclusion

MAb-based tumour-targeting therapy has proven to be an efficacious strategy for cancer treatment. Efforts including decreasing the immunogenicity of the mAb, improving the linker efficiency and enhancing drug potency have led to several immunoconjugates with impressive therapeutic results. One of them (Mylotarg) was appproved by the FDA and some others are in human clinical studies [8-11,106,122].

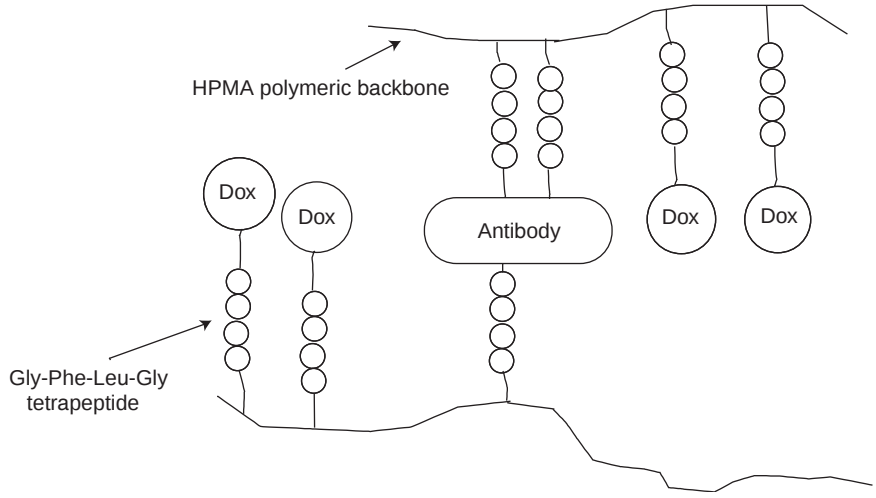


Figure 13. HPMA-based immunoconjugate with a classical structure [127,128]**.** Reprinted from ULBRICH K, SUBR V, STROHALM J, PLOCOVA D, JELINKOVA M, RIHOVA B: Polymeric drugs based on conjugates of synthetic and natural macromolecules I. Synthesis and physicochemical characterization. *J. Control. Release* **64**(1-3):63-79, Copyright (2000), with permission from Elsevier.
Dox: Doxorubicin; HPMA: *N*-(2-Hydroxypropyl)methacrylamide.

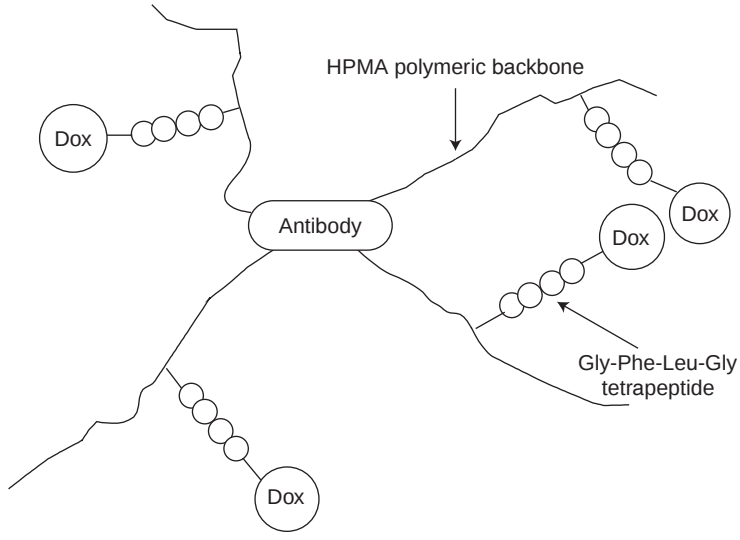


Figure 14. HPMA-based immunoconjugate with a star structure [127,128]**.** Reprinted from ULBRICH K, SUBR V, STROHALM J, PLOCOVA D, JELINKOVA M, RIHOVA B: Polymeric drugs based on conjugates of synthetic and natural macromolecules I. Synthesis and physicochemical characterization. *J. Control. Release* **64**(1-3):63-79, Copyright (2000), with permission from Elsevier.
Dox: Doxorubicin; HPMA: *N*-(2-Hydroxypropyl)methacrylamide.

However, there are still several limitations in this appoach:

- The identification of antigens, which are exclusively over-expressed in tumour tissues, is critical. In many cases, the antigens are also expressed in the normal tissues leading to diminished selectivity.
- Despite impressive progress in mAb technology, immunogenicity still represents a potential pitfall. MAb technology to generate humanised mAb has a greatly decreased immune response against HAMA. The use of human mAb is expected to solve this problem. Recent advances in polymer-based immunoconjugates introduced the benefits of the EPR effect; however, the addition of a polymer may elicit an unexpected immune response other than to the mAb.
- The large size of the mAb often results in poor penetration of immunoconjugates into solid tumours. Truncated mAb fragments have been investigated and improved penetration ability was observed.

Nevertheless, the target specificity achieved by mAb–cytotoxic agent conjugates is by far superior to any other tumour-targeting method currently available. Therefore, further advances in cancer biology, biomarkers, identification of tumour-specific antigens, protein engineering and mAb technology as well as progress in linker technology will undoubtedly facilitate and promote the applications of mAb-based immunoconjugates in clinic.

Acknowledgements

Part of the works presented here has been supported by grants from the National Institutes of Health (GM427980 and CA103314 to I.O.). Generous support from Indena, SpA as well as a partial support from ImmunoGen, Inc. is gratefully acknowledged.

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