Cancer Immunol Immunother (2003) 52: 328–337
DOI 10.1007/s00262-002-0352-9

SYMPOSIUM IN WRITING

Pamela A. Trail · H. Dalton King · Gene M. Dubowchik

Monoclonal antibody drug immunoconjugates for targeted treatment of cancer

Received: 26 August 2002 / Accepted: 3 October 2002 / Published online: 16 January 2003

Abstract Monoclonal antibodies (mAb) directed to tumor-associated antigens (TAA) or antigens differentially expressed on the tumor vasculature have been covalently linked to drugs that have different mechanisms of action and various levels of potency. The use of these mAb immunoconjugates to selectively deliver drugs to tumors has the potential to both improve antitumor efficacy and reduce the systemic toxicity of therapy. Several immunoconjugates, particularly those that incorporate internalizing antibodies and tumor-selective linkers, have demonstrated impressive activity in preclinical models. Immunoconjugates that deliver doxorubicin, maytansine and calicheamicin are currently being evaluated in clinical trials. The feasibility of using immunoconjugates as cancer therapeutics has been clearly demonstrated. Gemtuzumab ozogamicin, a calicheamicin conjugate that targets CD33, has recently been approved by the Food and Drug Administration (FDA) for treatment of acute myelogenous leukemia (AML). This review concentrates on the properties of the tumor and the characteristics of the mAb, linker, and drugs that influence the efficacy, potency, and selectivity of immunconjugates selected for cancer treatment.

Keywords Cancer · Cancer immunoconjugate · Immunotherapy · Monoclonal antibody

This article forms part of the Symposium in Writing on "Antibodies in Cancer Immunotherapy", published in this issue (Vol. 52) of the journal

P.A. Trail (✉)
West Haven, CT 06516–4175, USA
E-mail: pamela.trail.b@bayer.com

H.D. King (✉)
Wallingford, CT, 06492, USA
E-mail: dalton.king@bms.com

G.M. Dubowchik (✉)
Wallingford, CT, 06492, USA
E-mail: gene.dubowchik@bms.com

Introduction

The majority of drugs currently used to treat cancer are limited by a number of factors including the low therapeutic index of most chemotherapeutic agents, the emergence of drug- and radiation-resistant populations, tumor heterogeneity and the presence of metastatic disease. The current goal of cancer drug discovery and development is to identify agents that are effective cancer therapeutics and yet have minimal systemic side effects. One means to improve the selectivity of cancer therapy is by directing activity against therapeutic targets that display altered levels of expression on malignant versus normal cells. There has been substantial effort to rationally design these types of therapeutics both as low molecular weight compounds and as protein-based therapeutics such as monoclonal antibodies (mAb).

Significant progress has been made in the use of mAb as cancer therapeutics. Most notably there are now 4 mAb-based therapies approved by the Food and Drug Administration (FDA) for the treatment of cancer. The progress in antibody (Ab)-based therapy has largely resulted from Ab engineering technologies that yielded chimeric (mouse/human) or fully humanized mAb with reduced immunogenicity. Both chimerized and humanized mAb can be successfully administered to patients for a prolonged treatment duration without inducing a clinically meaningful immune response [1, 2, 3].

Chimeric and humanized mAb directed against tumor-associated antigens (TAA) have shown utility as monotherapy in the treatment of hematologic malignancies [4, 5]. mAb have also shown clinical activity when used as monotherapy in the treatment of solid tumors; however, activity is typically improved when the mAb are used in combination with cytotoxic drugs. mAb to HER2/neu used in combination with cisplatin [6] or paclitaxel [7] and mAb to the epidermal growth factor receptor (EGFR) when used in combination with cisplatin or doxorubicin (DOX) [8] have shown

improved response rates relative to those seen when the mAb are used as monotherapy [7]. The physical barriers associated with large solid tumors, including elevated interstitial pressure, heterogeneous and reduced functional vasculature and lymphatics as well as the relatively large distances for mAb to travel in the tumor interstitium [9] contribute to the limited tumor penetration and minimal efficacy seen when mAb-directed therapies are used as single agents in patients with advanced disease. In addition, antigenic heterogeneity both within a given histologic tumor type and within a given tumor can substantially affect mAb-directed therapies.

Immunoconjugates are bifunctional molecules that consist of a "targeting" domain that localizes in tumors coupled to a therapeutic moiety. Immunoconjugates, in the broadest definition, may utilize mAb, mAb fragments, hormones, peptides or growth factors to selectively localize cytotoxic drugs, plant and bacterial toxins, enzymes, radionuclides, photosensitizers, or cytokines to antigens expressed on tumor cells or on cells of the tumor neovasculature. This review will concentrate on the results obtained with mAb-drug immunoconjugates directed to tumor associated and vascular antigens. The properties of the tumor and the characteristics of the immunoconjugate that affect the efficacy, potency, and selectivity of this treatment modality will be discussed.

mAb and tumor-selective drug release

The use of immunoconjugates directed against TAA provides a means to improve efficacy by increasing the intratumoral concentration of the targeted drug. In addition, when a tumor-selective drug release mechanism is included in conjugate design, immunoconjugate therapy may reduce systemic toxicity. The clinical efficacy of mAb-directed therapy may be limited by expression of the targeted antigen on normal as well as malignant cells. For the most part, tumor-specific mAb have not been identified; rather, mAb identify TAA expressed at higher density on malignant cells relative to normal cells. Because the target antigen is likely to be expressed on cells of normal tissues it is important to balance the relative selectivity of the mAb with the potency of the agent delivered. It is also important to consider the systemic stability of the linker in the context of drug potency, the more potent the drug the more critical linker stability will be to safety.

In addition to immunoconjugates directed at TAA, several recent studies have evaluated the utility of targeting antigens expressed on the tumor neovasculature [10, 11, 12]. Directing therapy to the accessible vascular compartment may reduce the impact of barriers present in solid tumors that restrict mAb penetration and distribution [13]. Because of these factors, immunoconjugates directed against antigens differentially expressed on the tumor endothelium may offer potential advantages over targeting TAA expressed on cells of solid tumors.

Several conjugation strategies have been utilized in which the drug is attached to Ab that do not internalize following antigen-specific binding. To be effective these immunoconjugates must remain stable in the circulation and yet release active drug extracellularly following tumor localization. These immunoconjugates may rely on extracellular cleavage of peptidyl linkers [14] by enzymes such as cathepsins [15] and matrix metalloproteinases expressed by tumor cells [16], or rely on adventitious hydrolysis that may occur at the slightly acidic pH found in many solid tumors [17, 18]. For the most part, immunoconjugates that rely upon extracellular drug release demonstrate poor antigen-specific activity in vitro and in vivo [14, 19, 20].

One means to further improve the selectivity, efficacy, and potency of immunoconjugates is to design linkers that release active drug following endocytosis into target cells. These immunoconjugates utilize mAb that localize in the tumor and internalize into tumor cells following antigen-specific binding. Internalization of Ab [21] may occur rapidly (minutes to hours) or slowly (over several days). Because of the difference in internalization rate and intracellular fate seen for various Ab it is necessary to consider the rate of internalization in linker design. Ab that are rapidly internalized likely enter clathrin-coated pits and are internalized by receptor-mediated endocytosis. A variety of immunoconjugates have been shown to enter endosomes and lysosomes following antigen-specific binding and internalization [22, 23, 24, 25]. Linker strategies have been developed that exploit the relatively acidic pH or enzymatic content of lysosomes [23, 26, 27, 28, 29, 30]. These immunoconjugates utilize the Ab to direct the conjugate to antigen-expressing target cells and contain a linker designed to be stable in the circulation and to release biologically active drug only upon antigen-specific internalization. The extracellular stability and intracellular cleavage mechanism of the linker need to be considered in the context of the potency of the drug that is delivered; the more potent the drug, the greater is the requirement for extracellular stability.

mAb-directed delivery of anthracyclines

Immunoconjugates that utilize the anthracycline family of antitumor antibiotics have been extensively studied [19]. DOX is of particular interest for Ab-directed delivery, as it is a drug that has been shown clinically to have a broad spectrum of activity and to have toxic side effects that are both dose-related and predictable. The efficacy of DOX is limited by myelosuppression and cardiotoxicity [31], and as such the use of mAb-directed delivery should improve the efficacy by increasing the intratumoral accumulation of DOX while reducing systemic exposure. In addition, as DOX is well tolerated by patients, unanticipated systemic drug release from an immunoconjugate is not likely to result in unmanageable toxicity.

Acid-labile linkers

An acid-labile *cis*-aconityl linker was used to link DOX to the anti-melanoma mAb 9.9.27 [32]. The 9.9.27-DOX conjugate demonstrated antigen-specific activity in vitro and was active against DOX-insensitive melanoma xenografts. Following a 2-h exposure, intracellular DOX was detected in the cytoplasm and nuclei of antigen-expressing cells. When pH was increased by preincubation with chloroquine, cytoplasmic but not nuclear DOX was observed, demonstrating that the *cis*-aconityl linker released DOX in the acidic environment of lysosomes. The *cis*-aconityl linker was shown to have utility for linking both DOX and the anthracycline daunorubicin (DNR) to several mAb [32, 33, 34, 35, 36].

Hurwitz et al. [37] demonstrated the utility of attaching DNR to macromolecules using acid labile hydrazone linkages. In these studies conjugates that were prepared with acid-labile hydrazones were active in vitro, whereas those produced with a non-hydrolyzable linker were not. Similar results were obtained with morpholino-DOX (morphDOX), a DOX derivative that is 10–1,000 fold more potent than DOX. mAb LM609, an antibody that recognizes the intergrin $\alpha v\beta 3$ expressed on human melanoma cells, was conjugated to morph-DOX via a hydrazone or oxime linker. The in vitro cytotoxicity of these conjugates corresponded to their hydrolysis rates; conjugates containing the hydrazone linker were labile at pH 4.5 and demonstrated potent activity in vitro, while conjugates containing the oxime linker were stable at pH 4.5 and were not potent in cytotoxicity assays [38].

Other acid-sensitive conjugates of DNR/DOX have been obtained through modification of their C-13 carbonyl group to give hydrazones, acylhydrazone, semicarbazones, thiosemicarbazones, and oximes [39, 40, 41, 42]. A hydrazone linker was also used to conjugate DOX using a disulfide link to antilymphoma mAb. The conjugates obtained had drug/mAb molar ratios (MR) of 2–10, released DOX at acidic pH [43], and displayed antigen-specific cytotoxicity in vitro and in vivo against non-Hodgkin's lymphoma xenografts in athymic mice. The immunoconjugates produced antitumor activity that was better than that achieved with a matching dose of unconjugated DOX, and which was similar to that obtained with unconjugated DOX administered at its maximum tolerated dose (MTD) [41].

A similar strategy was used to produce immunoconjugates directed against a rapidly internalizing carcinoma antigen [23]. Conjugates were prepared with the anticarcinoma mAb BR64 which identifies a Le^y related TAA expressed at high density on the surface of cells from lung, colon, and breast [44]. The BR64 mAb was conjugated to DOX using a disulfide linker to the mAb and an acid-labile acyl-hydrazone linker to DOX. The BR64-DOX conjugates demonstrated antigen-specific activity in vitro and their potency was related to the DOX/mAb MR. Antigen-specific antitumor activity was also seen when BR64-DOX was evaluated against established human lung tumor xenografts in athymic mice. At higher dose levels, BR64-DOX treatment induced antitumor activity that was superior to that of unconjugated DOX administered at its MTD, unconjugated BR64 mAb, or mixtures of mAb and free DOX. In contrast to other treatment regimens, tumor regression and cure were obtained following treatment with BR64-DOX conjugates. However, regression was only seen at doses of BR64-DOX that approached its MTD. Thus, while the BR64-DOX conjugates demonstrated antigen-specific targeting and were more active than optimized unconjugated DOX, the therapeutic index of the conjugates was low, even against DOX-sensitive lung tumors [23]. The low potency of the disulfide linked BR64-DOX conjugates may have resulted from poor mAb localization, inefficient intracellular release of DOX, and/or systemic instability of the disulfide linker.

Since it was reasonable to expect that the disulfide linkage might not be sufficiently stable in the circulation, a systematic comparison of BR64-DOX immunoconjugates [45] was performed in which the only variable was the mAb-DOX linker. In these studies BR64 was conjugated to DOX (MR $\sim$8) using an acid-labile hydrazone linkage and either a disulfide or thioether bond to the mAb. Both the disulfide- and thioether-linked conjugates were generated from mild and selective opening of endogenous, interchain disulfides on the mAb [45]. The thioether-linked conjugate demonstrated substantially better stability in vitro. It is likely that immunoconjugates that demonstrate poor stability in vitro will also lack the metabolic stability needed for tumor localization and drug delivery. The data on the in vivo stability of the disulfide and thioether linked BR64 conjugates support this concept. The plasma terminal $T_{1/2}$ of conjugate-bound DOX was 30 h for the thioether-linked BR64 conjugate and only 17 h for the disulfide-linked conjugate. The total systemic (nontargeted) exposure to DOX, representing unwanted drug release, as assessed by plasma area under curve (AUC) values was 4-fold higher for the disulfide-linked conjugates. The increased stability of the thioether-linker resulted in almost a 10-fold increase in the amount of DOX delivered to tumors relative to that obtained with the disulfide-linked BR64 conjugate. The BR64 thioether conjugate was antigen-specific and both more potent and more active than either the BR64 disulfide conjugate or optimized unconjugated DOX against DOX-sensitive tumors. The superiority of the thioether-linked conjugate was even more striking when evaluated against a DOX-insensitive human colon tumor xenograft. Targeted delivery of DOX using the thioether linker resulted in substantial antitumor activity, including the cure of established colon tumors that were insenstitive to both unconjugated DOX and the disulfide-linked BR64-DOX conjugate [45]. It is likely that the increased stability of the thioether conjugate reflects its reduced susceptibility to extratumoral reductive agents such as glutathione or other thiol-containing

molecules in the plasma or liver. These studies clearly demonstrate the advantages of optimizing the systemic stability as well as the tumor-selective drug-releasing capacity of mAb-drug linkers used for internalizing immunoconjugates.

The BR64 mAb was not suitable for clinical development as it demonstrated binding to cardiac tissue in some patients. A closely-related mAb termed BR96 [44] that identifies a Le^y related TAA expressed at high density on the majority of human breast, lung and colon carcinomas but lacks cardiac reactivity, was selected for clinical development [44, 46, 47]. BR96 is a chimeric (mouse-human) mAb of the human IgG_1 isotype that is rapidly internalized following antigen-specific binding [48]. The BR96-DOX immunoconjugates contained a thioether linker to the mAb and an acyl hydrazone linker to DOX. Immunoconjugates were prepared by selectively reducing the 4 disulfide bonds in the hinge region of the mAb using dithiothreitol (DTT) followed by conjugation with the 6-(maleimidocaproyl)hydrazone of DOX [42, 46, 49]. The BR96-DOX conjugates produced antigen-specific activity in vitro, and potency was related to the density of BR96 expressed [46]. BR96-DOX demonstrated antigen-specific antitumor activity against established human breast, colon, and lung carcinoma xenografts in athymic mice. BR96-DOX, administered at tolerated doses, produced complete regression and/or cure of established DOX-sensitive lung tumors and displayed an excellent therapeutic index in preclinical models. When administered at 5% of its MTD, BR96-DOX was as active as optimized free DOX and when administered at 25% of its MTD, it produced cure in of 70% of mice bearing DOX-sensitive lung tumors [19, 46]. When evaluated against established colon tumor xenografts, BR96-DOX produced complete tumor regression and cure, even though the tumors were not sensitive to unconjugated DOX. In the case of the DOX-insensitive colon tumors, the dose of BR96-DOX required to produce regression was approximately twice that required for the DOX-sensitive lung tumor. BR96-DOX was both more potent and more active than DOX in each tumor model evaluated [19, 46].

Activity in models of metastatic and/or disseminated disease is an important criterion for evaluating the efficacy of immunoconjugate therapy. BR96-DOX therapy resulted in cure of 70% of mice bearing a large burden of disseminated disease ($\sim$0.5 g visible tumor burden). The median survival time of BR96-DOX-treated mice was significantly increased (>200 days) relative to that of untreated mice (90 days) or mice treated with the MTD of unconjugated DOX (94 days) [19, 46].

In many preclinical studies of immunoconjugates, the target antigen is tumor-specific as it is expressed on the human tumor but not on normal murine tissues. In rats as in humans, the BR96 mAb binds to differentiated cells of the gastrointestinal tract (stomach, esophagus, and intestine) as well as to acinar cells of the pancreas. Preclinical studies performed with human tumors in athymic rats and syngeneic BR96 expressing rat tumors in immunocompetent rats demonstrated complete regression and cure of tumors at tolerated doses, even when the BR96-defined antigen was expressed in some normal tissues [46, 50]. Interestingly, the BR96-DOX conjugate was also shown to be highly effective and well tolerated when administered to rats in a human lung cancer brain xenograft model [51].

The immunoconjugate BR96-DOX [42, 46]was evaluated in phase I [52] and II [53] clinical trials. Although cures were seen in multiple preclinical models, only tumor stabilization and a small number of partial regressions were seen in a phase I trial of patients with advanced disease. A therapeutically relevant anti-conjugate response was not observed and there was no significant hematologic or cardiac toxicity. Acute gastrointestinal toxicity with dose-related nausea, vomiting and gastritis was found to be dose-limiting [52]. A randomized phase II trial was performed in patients with metastatic breast carcinoma [53]. In this study, patients received 700 mg/m^2 of BR96-DOX (20 mg/m^2 DOX) or 60 mg/m^2 of DOX every 3 weeks. There was 1 partial response (in a patient with hepatic metastases) in the 14 patients receiving BR96-DOX and 1 complete and 3 partial responses in the 9 patients receiving DOX. Interestingly, 2 out of the 4 patients who crossed over to the BR96-DOX arm of the trial after persistent stable disease during DOX treatment achieved partial regression of hepatic metastases following BR96-DOX therapy.

Localization of BR96 and DOX was seen in tumor biopsies of patients receiving BR96-DOX, indicating that immunoconjugate had successfully delivered DOX to tumors [52]. These data, taken together with the low clinical response rates, suggest that the dose that could be safely administered every 3 weeks was insufficient to maintain the intratumoral concentration of DOX required to achieve tumor regression.

Because of the physical barriers preventing efficient diffusion of high molecular weight drugs into large tumors, it is likely that immunoconjugate therapy will be most successful when used to treat patients with minimal residual disease and when used in combination with other therapeutic modalities. Preclinical studies were performed with BR96-DOX to determine whether it was possible to reduce the dose level of conjugate required for efficacy by combination therapy with paclitaxel [54, 55]. Combined therapy with BR96-DOX and paclitaxel resulted in a dramatic increase in antitumor activity against human tumors in athymic mice and rats [54]. This was true even when BR96-DOX was evaluated at doses that were not active as single agents. A significant increase in antitumor activity was seen for the combination relative to that of equivalent doses of BR96-DOX and paclitaxel administered alone in 4 tumor xenografts of 3 histologic types: breast, lung, and colon carcinoma. The antitumor activity achieved with combined paclitaxel and BR96-doxorubicin therapy was observed both when paclitaxel was administered at its MTD and at suboptimal doses. BR96-DOX in combination with paclitaxel resulted in increased antitumor activity

against paclitaxel-sensitive lung and breast tumor xenografts and also paclitaxel-insensitive colon tumors [54]. Studies performed in vitro demonstrated that treatment of BR96 expressing cells with BR96-DOX 24 h prior to treatment with paclitaxel resulted in an increase in G2 tumor cells that were more sensitive to paclitaxel treatment [55]. The potential advantage of combination therapy with the BR96-DOX conjugate, now termed SGN-15, and taxanes is currently being evaluated in clinical trials [56].

The potency of immunoconjugates can be improved by increasing the quantity of drug delivered per mAb [23, 57]. However, in the case of DOX immunoconjugates, significant losses in affinity and antigen-specific cytotoxicity were seen when $\geq$10 molecules of DOX were directly conjugated per mAb [57]. In another approach to increase the drug/mAb molar ratio, "branched" linkers in which each thioether-attached linker to the mAb carries 2 DOX molecules, resulted in an increase in the drug:mAb MR from 8 to 16. These conjugates contained $< 5\%$ aggregate, and retained $> 95\%$ binding activity relative to unconjugated mAb. The 2-fold increase in MR was accompanied by an increase in antigen-specific potency in vitro and importantly, a 2-fold decrease in the amount of mAb required to achieve partial regression of s.c. tumors in preclinical models [58].

Enzyme cleavable linkers

Immunoconjugates have also been designed with peptide linkers that are sensitive to cleavage by lysosomal [27, 29, 30, 59] or tumor-associated [14] enzymes. Immunoconjugates in which the internalizing mAb BR96 was conjugated to DOX through 2 lysosomally cleavable dipeptides, Phe-Lys and Val-Cit, required a self-immolative *p*-aminobenzyloxycarbonyl (PABC) spacer between the dipeptides and the DOX for efficient generation of free drug. Both conjugates demonstrated rapid and near-quantitative DOX release when incubated with either the cysteine protease cathepsin B or in a rat liver lysosomal preparation. Interestingly, both conjugates released DOX more rapidly in the lysosomal preparation than with cathepsin B alone, suggesting that other lysosomal proteases were able to cleave the dipeptide linkers. The BR96 dipeptide conjugates demonstrated excellent antigen-specific activity in vitro. Against BR96-expressing lung carcinoma cells both BR96-DOX conjugates were > 200-fold more potent than a non-binding IgG-DOX conjugate prepared using the same linker [59]. A corresponding immunoconjugate of the topoisomerase I inhibitor camptothecin (CPT), BR96-Phe-Lys-PABC-CPT (MR = 7.5) was also prepared [29] and demonstrated potent antigen-specific cytotoxicity, equivalent to free CPT, against human lung carcinoma cells.

Peptide-linked DOX immunoconjugates were also prepared using a polyethylene glycol (PEG)-based cleavable linker [60]. The PEG moiety was reported to increase conjugate solubility and monomeric integrity, 2 factors that are often adversely affected by attachment of hydrophobic cytotoxic agents and linkers. Conjugates were attached through several PEG-linked dipeptides; alanyl-proline, alanyl-valine, and glycyl proline to NL-1, an Ab that binds to CD10 (also termed common acute lymphoblastic leukemia antigen or CALLA). CD10 is over-expressed on several tumor types, is identical to neutral endopeptidase (NEP) [60], and mAb to CD10 are rapidly internalized following antigen-specific binding [61]. NL-1 conjugates (MR = 1–2) that utilized Ala-Val and Gly-Pro peptidyl linkers demonstrated antigen-specific cytotoxicity, whereas the Ala-Pro conjugate did not. The substrate selectivity seen for these peptides is interesting, particularly as extracellular DOX release from the alanyl-valine conjugate may be mediated through the endopeptidase activity of NEP, while intracellular release may be mediated by lysosomal proteases [6060]. It will be of interest to evaluate the antigen-selectivity of these conjugates in vivo to further understand the role of extracellular versus intracellular drug release from these peptidyl linkers.

In an attempt to further improve the potency of dipeptide-linked conjugates, a branching strategy similar to that used for hydrazone [58] linkers was employed [30]. Thioether-linked conjugates [42, 46] of the internalizing mAb BR96 were produced and DOX was coupled to a branched dipeptide linker designed to liberate DOX following antigen-specific internalization into lysosomes. The use of the branched linker resulted in approximately a 2-fold increase in DOX:mAb MR relative to the previously described non-branched peptide conjugates [59]. The branched peptide linkers rapidly released DOX when exposed to the lysosomal enzyme cathepsin B and the corresponding BR96 conjugates demonstrated potent antigen-specific cytotoxicity in vitro. However, the conjugates were shown by size exclusion chromotography to exist almost exclusively as a non-covalently associated dimers [59]. However, these conjugates were obtained as discrete monomers when a methoxytriethyleneglycol (mTEG) chain was attached to each DOX through an acid-cleavable hydrazone bond. The chain serves to disrupt the steric, hydrophobic, and pi-stacking interactions that apparently contribute to dimer formation. In tumor cell lysosomes, both acid-catalyzed hydrolysis of the mTEG groups and cathepsin B cleavage of the dipeptide linkers occurs to liberate DOX. On a mAb basis, the mTEG-modified branched BR96 peptide conjugates were more potent than single chain BR96 peptide conjugates and unmodified branched peptide BR96-DOX conjugates in vitro [62].

mAb-directed delivery of highly potent drugs

In addition to increasing the quantity of drug delivered by an Ab molecule, it is possible to substantially improve immunoconjugate potency and efficacy by increasing the potency of the targeted drugs. Maytansanoids inhibit tubulin polymerization and are

100–1,000-fold more potent than conventional cytotoxic drugs. The maytansanoids, although highly potent, have a poor therapeutic index because of their high systemic toxicity. The use of mAb-directed delivery affords a potential means to exploit the potency of maytansinoids against tumors while reducing their systemic toxicity. A maytansinoid derivative, DM1, was produced and attached through a disulfide linkage to mAb C242, a murine IgG_1 mAb that binds to a mucin-type glycoprotein expressed on human colorectal cancer cells. The MR obtained for C242-DM1 conjugates was typically 4. The C242-DM1 conjugates demonstrated antigen-specific activity in vitro and when evaluated against established tumor xenografts they were more active than a non-binding control DM1 conjugate or mixtures of mAb C242 and unconjugated DM1 [63]. The internalizing murine mAb 8D11 binds to prostate stem cell antigen (PSCA) expressed on primary and metastatic prostate tumors as well as differentiated luminal cells of the prostate. The 8D11 mAb was conjugated to DM1 using the same linker chemistry [63]. The 8D11-DM1 conjugates demonstrated antigen-specific activity in vitro. When evaluated against established human tumor xenografts expressing PSCA at high density, the 8D11-DM1 conjugates demonstrated impressive antigen-specific antitumor activity, including regression of large tumors at dose levels of 5.5 mg/kg mAb (75 μg/kg DM1). At the same dose level, the non-binding control mAb-DM1 conjugate produced a delay in tumor growth but not tumor regression. Antitumor activity was related to the density of antigen expressed. The 8D11-DM1 conjugate produced tumor growth inhibition but not tumor regression when evaluated against a prostate tumor, SW780, that expressed PSCA at low density. Interestingly, the 8D11-DM1 conjugate did not show antigen-specific activity against SW780 line when evaluated in vitro. It is not clear from these data whether the in vitro exposure time was not sufficient to produce cytotoxicity against a cell line expressing the targeted antigen at low density or whether the tumor growth delay seen in vivo results, at least in part, from adventitious drug release [64]. The potential utility of mAb delivery of maytansanoids is currently being evaluated clinically.

The enediyne family of antibiotics are among the most toxic antitumor compounds described to date. This novel class of agents includes the calicheamicins, neocarzinostatin, esperamicins, dynemicins, kedarcidin, and maduropeptin [65]. These agents are highly potent in vitro, efficiently producing double-stranded DNA breaks at very low drug concentrations. However, as with the maytansinoids described above, the utility of the enediynes as antitumor drugs has been limited by their low therapeutic index. The use of mAb to selectively deliver enediyenes provides a means to exploit the impressive potency of these compounds while limiting their systemic toxicity. The use of extremely toxic drugs requires both careful mAb selection, as even low levels of expression of the targeted antigen by normal cells may lead to unacceptable toxicity, and a linker that is metabolically stable and yet releases drug selectively following tumor localization. Neocarzinostatin [66, 67, 68, 69] and several of the calicheamicins [25, 70, 71, 72] have been used to produce extremely potent immunoconjugates. In fact, the first mAb-drug immunoconjugate to be approved by the FDA consists of a calicheamicin analog conjugated to an antibody directed against CD33. This conjugate has been referred to as CMA-676, gemtuzumab ozogamicin, and mylotarg.

Calicheamicin conjugates were produced by periodate oxidation of the carbohydrate residues of the internalizing anti-polyepithelial mucin mAb CT-M-01 followed by reaction with a hydrazide derivative of calicheamicin γ^{I}_{1}. These "carbohydrate" conjugates utilize an acid labile hydrazone bond to the mAb to insure hydrolysis following internalization into lysosomes, and a sterically protected disulfide bond to the calicheamicin to increase stability in circulation. The CT-M-01 conjugates demonstrated potent antigen-specific activity against subcutaneous breast tumor xenografts in athymic mice [25].

mAb P67.6 binds to CD33, a 67-kDa glycoprotein expressed on the surface of leukemic cells of patients with acute myelogenous leukemia (AML). The CD33 antigen is rapidly internalized following antigen-specific binding. Importantly, the CD33 antigen is not expressed by immature pluripotent stem cells even though it is expressed on normal progenitor and mature myeloid cells [72]. Calicheamicin conjugates of mAb P67.6 were prepared as described previously [25]. These conjugates, referred to as "carbohydrate" conjugates, contained the acid-labile hydrazone bond. Immunoconjugates of mAb P67.6 were also prepared that contained a hydrolytically stable amide bond. These "amide" conjugates relied on cleavage of the disulfide present in all calicheamicin conjugates as the mechanism of drug release. The MR of the carbohydrate and amide conjugates was similar, typically 2–3. The hydrazone linked carbohydrate conjugates of P67.6 were shown to be superior to the amide-linked conjugates in terms of antigen-specific activity in vitro. In addition, treatment with tolerated doses of the carbohydrate conjugate resulted in complete regressions of established antigen-expressing tumors whereas the amide conjugate produced only a delay in tumor growth suggesting that the hydrazone linker provided a more efficient, tumor selective, mechanism of drug release [73].

Although successful for murine P67.6, the carbohydrate linker strategy could not be used with the humanized (human IgG_4) P67.6 mAb (hP67.6) since the required periodate treatment resulted in a substantial loss of immunoreactivity and antigen-specific potency [74]. Several bifunctional linkers were evaluated that utilized lysine attachment to the mAb via amide bonds and incorporated an acid-labile hydrazone bond to liberate calicheamicin following antigen-specific internalization. The use of an acetylphenoxy(butanoic acid) linker was most successful, and yielded hP67.6 conjugates with a drug:mAb molar ratio of 2–3. This hP67.6

calicheamicin conjugate, given the generic name of gemtuzumab ozogamicin, produced antigen-specific cytotoxicity in vitro and in vivo. Gemtuzumab ozogamicin was shown to be more potent in vivo than the carbohydrate-based murine P76.6 conjugate and also displayed greater selectivity in vitro. The therapeutic index of the conjugate was at least 6 when evaluated against established tumors in athymic mice [74].

A phase I study of gemtuzumab ozogamicin (referred to as CMA-676) was performed in patients with AML [72]. Forty patients with refractory or relapsed AML were treated with 0.25–9.0 mg/m^2 of gemtuzumab ozogamicin. Toxicity was primarily hematologic; however, hematologic side effects were not considered dose-limiting. Fever and chills occurred in 80% of patients, and were the most common non-hematologic side effect. Leukemic cells were eliminated from the blood and marrow of 20% of treated patients. At the 9 mg/m^2 dose level, $>75\%$ saturation of CD33 sites was seen on peripheral blood blast cells. Clinical responses were seen at dose levels of 1–9 mg/m^2. Responses were seen only in patients whose peripheral blast cells had a low efflux of 3,3'-diethyloxacarbocyanine iodide and demonstrated $\geq$75% saturation of CD33. The efflux data suggest that intracellular delivery of calicheamicin by the P67.6 mAb did not overcome multi-drug resistance [72].

Three open-label multicenter phase II trials that enrolled a total of 142 adult patients with AML in first relapse were conducted to evaluate the efficacy and safety of gemtuzumab ozogamicin (also referred to as CMA-676). Patients received a 2-h infusion of 9 mg/m2 gemtuzumab ozogamicin administered every 2 weeks for a total of 2 doses. Thirty percent of the patients achieved clinical remission. The major toxicity was hematologic, as would be expected from CD33-directed cytotoxic therapy. Non-hematologic side effects occurred in a minority of patients and included grade 3 or 4 hyperbilirubinemia, elevated liver transaminase levels, mucositis, and infections [71]. The extent of liver toxicity seen in a subset of 23 patients that received gemtuzumab ozogamicin for AML following hematopoietic cell transplant was evaluated [75]. Eleven of the 23 patients developed liver injury and 7 of these patients died with persistent liver dysfunction. Histologic evaluation revealed deposition of sinusoidal collagen. This type of liver toxicity may result from targeted delivery of calicheamicin to CD33-expressing cells found in hepatic sinusoids [75].

The in vivo binding and internalization of gemtuzumab ozogamicin was also evaluated during a phase II trial of patients with AML in first relapse. Patients received a 2-h infusion of 9 mg/m2 gemtuzumab ozogamicin. Within 3 h of the start of infusion, nearly complete saturation of CD33 antigenic sites was observed for circulating leukemic and normal myeloid cells. The conjugate was shown to be rapidly internalized by leukemic and normal CD33 expressing myeloid cells and to result in apoptosis of these cells but not CD33 negative lymphoid cells [76].

The development of several other calicheamicin derivatives for use in mAb-directed delivery has been described. Calicheamicin θ^{I}_{1} (Camθ) a more potent synthetic analog of calicheamicin γ^{I}_{1}, was conjugated through a disulfide linkage to mAb 14G2a, an anti-ganglioside GD2 mAb. The 14G2a-Camθ conjugate showed impressive antitumor activity when used to treat experimental liver metastases in syngeneic immunocompetent mice [70]. Dose-dependent antitumor activity was observed against a neuroblastoma line with heterogeneous antigen expression. The 14G2a-Camθ conjugate was both more effective and less toxic than unconjugated Camθ or mixtures of mAb 14G2a and unconjugated Camθ, indicating effective mAb-directed targeting. The use of a syngeneic tumor with heterogeneous expression of the target antigen more closely approximates the clinical situation and provides an important model system for evaluating immunoconjugate efficacy.

The calicheamicin derivative Camθ was also conjugated though a disulfide linkage to mAb 138H11, a mAb directed against human gamma glutamyl transferase. The antigen identified by mAb 138H11 is expressed on cells of normal kidney and liver. However, in normal organs the antigen is not accessible via the circulation as it is expressed on the luminal surface of the liver bile canaliculi and the brush border membrane of the promixal tubules of the kidney. In primary and metastatic renal cell carcinomas, the antigen is expressed over the entire surface and is thus accessible to circulating mAb. The 138H11-Camθ conjugate demonstrated potent antigen specific activity in vitro when evaluated against the Caki-1 renal cell carcinoma line. The EC_{50} of the conjugate was 5×10^{-11} M, approximately 40-fold more potent than a non-binding control Camθ conjugate. The 138H11-Camθ conjugate demonstrated antigen-specific activity when administered to athymic mice on the day of tumor implant. The 138H11-Camθ conjugate administered at a dose of 20 μg/kg Camθ was more active than unconjugated Camθ, mAb 138H11, or mixtures of mAb and unconjugated Camθ. However, the conjugate was quite toxic, producing 23% body weight loss and 5/9 deaths. These data suggest that the disulfide linker was not stable enough in circulation to selectively deliver this potent calicheamcin analog to tumors [77].

Targeting the tumor vasculature

The progressive growth and metastatic dissemination of solid tumors requires the formation of new blood vessels (angiogenesis) from the pre-existing vasculature [78]. Several studies have demonstrated that targeting the tumor vasculature with mAb [11, 79, 80], growth factor ligands [81, 82], or ligands which bind $\alpha v\beta 3$ integrins [10, 12] can produce impressive antitumor activity. Directing immunoconjugate therapy to antigens differentially expressed on the tumor endothelium offers several

potential advantages over targeting TAA expressed on cells of solid tumors. Targeting the accessible vascular compartment may reduce the impact of physical barriers of solid tumors, such as elevated interstitial pressure and heterogeneous blood flow, that restrict the penetration and distribution of mAb through the tumor parenchyma [9]. Furthermore, endothelial cells are highly regulated, genetically stable cells that are less likely to develop the classical drug resistance observed in tumor cells [78]. As angiogenesis is required for tumor progression, therapies directed against the tumor vasculature should have broad spectrum antitumor activity and the therapeutic effect should be amplified as each blood vessel supports the growth of 5–100 tumor cells [78]. The identification of appropriate target antigens that are expressed on the tumor neovasculature but not on cells of normal vessels is an area of ongoing interest. Potential antigens for vascular targeting include vascular endothelial growth factor receptor (VEGFR-2), endoglin, endosialin, aminopeptidase A [11, 80, 83], VEGF complexed with its receptor [84], and the $\alpha v\beta 3$ integrins [10, 12]. The in vivo screening of phage peptide libraries is an ongoing approach to identify novel molecules expressed on angiogenic blood vessels. As with all targeted approaches, the safety and overall success of therapies that target the tumor vasculature will require an appropriate balance between the selectivity of expression of the antigenic target and the potency and mechanism of action of the targeted agent.

Conclusions and future directions

The approval of several mAb and a mAb-calicheamicin immunoconjugate make it clear that the promise of mAb-directed therapies has begun to be realized. Additional advances in this therapeutic approach are aimed at improving the efficacy and therapeutic index of immunoconjugates by optimizing the selectivity of the targeting mAb and the potency of the targeted drug. One area of particular interest is in the design of linkers that are truly stable in the circulation and yet liberate drug following internalization into tumor cells. These types of linker modifications may improve the side effect profile of immunoconjugates, but perhaps more importantly, by exploiting the long circulating half-life of chimerized and humanized mAb, substantial improvements may be expected in the efficacy of immunoconjugates. In addition to research efforts directed at improving the design of immunoconjugates, clinical studies are underway that are beginning to define optimal therapeutic strategies for these agents. In the case of solid tumors, where immunoconjugate penetration and distribution remains a substantial challenge, it is likely that the major clinical role for immunoconjugates will be in combination with small molecule chemotherapeutic agents and when used in minimal disease settings.

References

1. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L (1999) Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. Semin Oncol 26:78
2. Davis T, Grillo-Lopez AJ, White CA, McLaughlin P, Czuczman MS, Link BK, Maloney DG, Weaver RL, Rosenberg J, Levy R (2000) Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of retreatment. J Clin Oncol 18:3135
3. Saleh MN, Khazaeli MB, Grizzle WE, Wheeler RH, Lawson S, Liu T, Russell C, Meredith R, Schlom J, LoBuglio AF (1993) A phase I clinical trial of murine monoclonal antibody D612 in patients with metastatic gastrointestinal cancer. Cancer Res 53:4555
4. Huhn D, von Schilling C, Wilhelm M, Ho AD, Hallek M, Kuse R, Knauf W, Riedel U, Hinke A, Srock S, Serke S, Peschel C, Emmerich B (2001) Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. Blood 98:1326
5. Grillo-Lopez AJ, White CA, Dallaire BK, Varns CL, Shen CD, Wei A, Leonard JE, McClure A, Weaver R, Cairelli S, Rosenberg J (2000) Rituximab: the first monoclonal antibody approved for the treatment of lymphoma. Curr Pharm Biotechnol 1:1
6. Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga MM, Tripathy D, Baly D, Baighman SA, Twaddell T, Glaspy JA, Slalom DJ (1998) Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol 16:2659
7. Baselga J (2001) Herceptin alone or in combination with chemotherapy in the treatment of HER2-positive metastatic breast cancer: pivotal trials. Oncology 61(2):14
8. Baselga J (2001) The EGFR as a target for anticancer therapy – focus on cetuximab. Eur J Cancer 37(4):S16
9. Jain RK (1998) Delivery of molecular and cellular medicine to solid tumors. J Controlled Release 53:49
10. Arap W, Pasqualini R, Ruoslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377
11. Huang X, Molema G, King S, Watkins L, Edgington TS, Thorpe PE (1997) Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. Science 275:547
12. Hood JD, Bednarski M, Frausto R, Guccione S, Reisfeld RA, Xiang R, Cheresh DA (2002) Tumor regression by targeted gene delivery to the neovasculature. Science 296:2404
13. Jain R (1994) Barriers to drug delivery in solid tumors. Sci Am 271:58
14. Suzawa T, Nagamura S, Saito H, Ohta S, Hanai N, Yamasaki M (2000) Synthesis of a novel duocarmycin derivative DU-257 and its application to immunoconjugate using poly(ethylene glycol)-dipeptidyl linker capable of tumor specific activation. Bioorg Med Chem 8:2175
15. Yano M, Hirai K, Naito Z, Yokoyama M, Ishiwata T, Shiraki Y, Inokuchi M, Asano G (2001) Expression of cathepsin B and cystatin C in human breast cancer. Surg Today 31:385
16. Nabeshima K, Inoue T, Shimao Y, Sameshima T (2002) Matrix metalloproteinases in tumor invasion: role for cell migration. Pathol Int 52:255
17. Evelhoch JL (2001) pH and therapy of human cancers. Novartis Found Symp 240:68
18. Murdter TE, Friedel G, Backman JT, McClellan M, Schick M, Gerken M, Bosslet K, Fritz P, Toomes H, Kroemer HK, Sperker B (2002) Dose optimization of a doxorubicin prodrug (HMR 1826) in isolated perfused human lungs: low tumor pH

promotes prodrug activation by beta-glucuronidase. J Pharmacol Exp Ther 301:223
19. Trail PA, Willner D, Hellstrom KE (1995) Site-directed delivery of anthracyclines for cancer therapy. Drug Dev Res 34:196
20. Dubowchik GM, Walker MA (1999) Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. Pharmacol Ther 83:67
21. Kyriakos RJ, Shih LB, Ong LG, Goldenberg DM, Mattes JM (1992) The fate of antibodies bound to the surface of tumor cells in vitro. Cancer Res 52:835
22. Arnon R, Sela M (1982) In vitro and in vivo efficacy of conjugates of daunomycin with anti-tumor antibodies. Immunol Rev 62:5
23. Trail PA, Willner D, Lasch SJ, Henderson AJ, Greenfield RS, King D, Zoeckler ME, Braslawsky GR (1992) Antigen specific activity of carcinoma reactive BR64-adriamycin conjugates evaluated in vitro and in human tumor xenograft models. Cancer Res 52:5693
24. Wargalla UC, Reisfeld RA (1989) Rate of internalization of an immunotoxin correlates with cytotoxic activity against human tumor cells. Proc Natl Acad Sci USA 86:5146
25. Hinman LM, Hamann PR, Wallace R, Menendez AT, Durr FE, Upeslacis J (1993) Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. Cancer Res 53:3336
26. Srinivasachar K, Neville DM Jr (1989) New protein cross-linking reagents that are cleaved by mild acid. Biochemistry 28:2501
27. Trouet A, Masquelier M, Baurain R, Deprez-de Compeneere D (1982) A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: in vitro and in vivo studies. Proc Natl Acad Sci USA 79:626
28. Shen WC, Ballou B, Ryser HJP, Hakala TR (1986) Targeting, internalization, and cytotoxicity of methotrexate-monolconal anti-stage-specific embryonic antigen-1 antibody conjugates in cultured F-9 teratocarcinoma cells. Cancer Res 46:3912
29. Walker MA, Dubowchik GM, Hofstead SJ, Trail PA, Firestone RA (2002) Synthesis of an immunoconjugate of camptothecin. Bioorg Med Chem Lett 12:217
30. Dubowchik GM, Radia S, Mastalerz H, Walker MA, Firestone RA, Dalton King H, Hofstead SJ, Willner D, Lasch SJ, Trail PA (2002) Doxorubicin immunoconjugates containing bivalent, lysosomally-cleavable dipeptide linkages. Bioorg Med Chem Lett 12:1529
31. Carter SK (1975) Adriamycin: a review. J Natl Cancer Inst 55:1265
32. Yang HM, Reisfeld RA (1988) Doxorubicin conjugated with a monoclonal antibody directed to a human melanoma-associated proteoglycan suppresses the growth of established tumor xenografts in nude mice. Proc Natl Acad Sci 85:1189
33. Gallego J, Price MR, Baldwin RW (1984) Preparation of four daunomycin monoclonal antibody 791T/36 conjugates with anti-tumor activity. Int J Cancer 33:737
34. Diener E, Diner UE, Sinha A, Xie A, Vergidis R (1986) Specific immunosuppression by immunotoxins containing daunomycin. Science 231:148
35. Dillman RO, Johnson DE, Shawler DL, Koziol JA (1988) Superiority of an acid-labile daunorubicin-monoclonal antibody conjugate compared to free drug. Cancer Res 48:6097
36. Hudecz F, Ross H, Price MR, Baldwin RW (1990) Immunoconjugate design: a predictive approach for coupling daunomycin to monoclonal antibodies. Bioconj Chem 1:197
37. Hurwitz E, Wilchek M, Pitha J (1980) Soluble molecules as carriers for daunorubicin. J Appl Biochem 32:25
38. Mueller BM, Wrasidlo WA, Reisfeld RA (1990) Antibody conjugates with morpholinodoxorubicin and acid cleavable linkers. Bioconj Chem 1:325
39. Yamamoto K, Acton EM, Henry DW (1972) Antitumor activity of some derivatives of daunarubicin at the amino group and methyl ketone functions. J Med Chem 15:872
40. Phillips DR, Baguley BC, Brownlee RT, Cacioli P, Chandler CJ, Kyratzis I, Reiss JA, Scourides PA (1990) The synthesis, stability and biological activity of bis-intercalating bis-daunomycin hydrazones. Drug Des Deliv 5:203
41. Braslawsky GR, Edson MA, Pearce W, Kaneko T, Greenfield RS (1990) Antitumor activity of adriamycin (hydrazone linked) immunoconjugates compared to free adriamycin and specificity of tumor cell killing. Cancer Res 506:6608
42. Willner D, Trail PA, Hofstead SJ, King HD, Lasch SJ, Braslawsky GR, Greenfield RS, Kaneko T, Firestone RA (1993) (6-Maleimidocaproyl)hydrazone of doxorubicin – a new derivative for the preparation of immunoconjugates of doxorubicin. Bioconj Chem 4:521
43. Greenfield RS, Kaneko T, Daues A, Edson MA, Fitzgerald KA, Olech L, Grattan JA, Braslawsky GR (1990) Evaluation in vitro of adriamycin – immunoconjugates synthesized using an acid sensitive hydrazone linker. Cancer Res 50:6600
44. Hellström I, Garrigues HJ, Garrigues U, Hellström KE (1990) Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le^y-related cell surface antigen. Cancer Res 50:2183
45. Trail PA, Willner D, Knipe J, Henderson AJ, Lasch SJ, Zoeckler ME, Trailsmith MD, Doyle TW, King HD, Casazza AM, Braslawsky GR, Hofstead SJ, Greenfield RS, Firestone RA, Mosure KW, Kadow KF, Yang MB, Hellström KE, Hellström I (1997) Effect of linker variation on the stability, potency, and efficacy of carcinoma reactive BR64-doxorubicin immunoconjugates. Cancer Res 57:100
46. Trail PA, Willner D, Lasch SJ, Henderson AJ, Hofstead SJ, Casazza AM, Firestone RA, Hellström I, Hellström KE (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science 261:212
47. Trail PA, Slichenmyer WJ, Birkhofer MJ, Warner G, Knipe J, Willner D, Firestone RA, Sikkema D, Onetto N, Canetta R, Saleh MN, Murray JL, Gilewski TA, Bookman MA, Hellstrom I, Hellstrom KE (1996) BR96-doxorubicin immunoconjugate for treatment of patients with carcinoma. Proc Am Assoc Cancer Res 37:626
48. Garrigues J, Garrigues U, Hellström I, Hellström KE (1993) Le^y specific antibody with potent anti-tumor activity is internalized and degraded in lysosomes. Am J Pathol 142:607
49. Firestone R, Firestone RA, Willner D, Hofstead SJ, King HD, Kaneko T, Braslawsky GR, Greenfield RS, Trail PA, Lasch SJ, Henderson AJ, Casazza AM, Hellström I, Hellström KE (1996) Synthesis and antitumor activity of the immunoconjugate BR96-DOX. J Controlled Release 39:251
50. Sjögren HO, Isaksson M, Willner D, Hellström I, Hellström KE, Trail PA (1997) Antitumor activity of carcinoma-reactive BR96-doxorubicin conjugate against human carcinomas in athymic mice and rats and syngeneic rat carcinomas in immunocompetent rats. Cancer Res 57:4530
51. Remsen LG, Trail PA, Hellstrom I, Hellstrom KE, Neuwelt EA (2000) Enhanced delivery improves the efficacy of a tumor-specific doxorubicin immunoconjugate in a human brain tumor xenograft model. Neurosurgery 46:704
52. Saleh MN, LoBuglio AF, Trail PA (1998) Monoclonal antibody-based therapy of solid tumors: studies with BR96-doxorubicin. In: Grossbard M (ed) Monoclonal antibody-based therapy of cancer. Marcel Dekker, New York, 397
53. Tolcher AW, Sugarman S, Gelmon KA, Cohen R, Saleh M, Isaacs C, Young L, Healey D, Onetto N, Slichenmyer W (1999) Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. J Clin Oncol 17:478
54. Trail PA, Willner D, Bianchi AB, Henderson AJ, Trailsmith MD, Girit E, Lasch S, Hellstrom I, Hellstrom KE (1999) Enhanced antitumor activity of paclitaxel in combination with the anticarcinoma immunoconjugate BR96-doxorubicin. Clin Cancer Res 5:3632
55. Wahl AF, Donaldson KL, Mixan BJ, Trail PA, Siegall CB (2001) Selective tumor sensitization to taxanes with the mAb-drug conjugate cBR96-doxorubicin. Int J Cancer 93:590
56. Nabell L, Saleh M, Marshall J, Hart L, O'Keefe C, Thornton J, Carlisle R, Lavelle P, Ley L, Malero-Jordan N, Sandler A,

Siegall C, LoBuglio A, Sing A (2002) Phase II study of SGN-15 (cBR96-doxorubicin immunoconjugate) combined with docetaxel for the treatment of metastatic breast and colorectal carcinoma. Proc Am Soc Clin Oncol 21:15a
57. Shih LB, Goldenberg DM, Xuan H, Lu H, Sharkey RM, Hall TC (1991) Anthracycline immunoconjugates prepared by a site specific linkage via an amino-dextran intermediate carrier. Cancer Res 51:4192
58. King HD, Yurgaitis D, Willner D, Firestone RA, Yang MB, Lasch SJ, Hellstrom KE, Trail PA (1999) Monoclonal antibody conjugates of doxorubicin prepared with branched linkers: a novel method for increasing the potency of doxorubicin immunoconjugates. Bioconjug Chem 10:279
59. Dubowchik G, Firestone R, Padilla L, Willner D, Hofstead S, Mosure K, Knipe J, Lasch S, Trail P (2002) Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. Bioconj Chem 13:855
60. Suzawa T, Nagamura S, Saito H, Ohta S, Hanai N, Kanazawa J, Okabe M, Yamasaki M (2002) Enhanced tumor cell selectivity of adriamycin-monoclonal antibody conjugate via a poly(ethylene glycol)-based cleavable linker. J Controlled Release 79:229
61. Pesando JM, Tomaselli KJ, Lazarus H, Schlossman SF (1983) Distribution and modulation of a human leukemia-associated antigen (CALLA). J Immunol 131:2038
62. King H, Dubowchik G, Mastalerz H, Willner D, Hofstead S, Firestone R, Lasch S, Trail P (2002) Monoclonal antibody conjugates of doxorubicin prepared with branched peptide linkers: inhibition of aggregation by methoxytriethyleneglycol chains. J Med Chem 45:4336
63. Liu C, Tadayoni BM, Bourret LA, Mattocks KM, Derr SM, Widdison WC, Kedersha NL, Ariniello PD, Goldmacher VS, Lambert JM, Blattler WA, Chari RV (1996) Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. Proc Natl Acad Sci USA 93:8618
64. Ross S, Spencer SD, Holcomb I, Tan C, Hongo J, Devaux B, Rangell L, Keller GA, Schow P, Steeves RM, Lutz RJ, Frantz G, Hillan K, Peale F, Tobin P, Eberhard D, Rubin MA, Lasky LA, Koeppen H (2002) Prostate stem cell antigen as therapy target: tissue expression and in vivo efficacy of an immunoconjugate. Cancer Res 62:2546
65. Smith AL, Nicolaou KC (1996) The enediyne antibiotics. J Med Chem 39:2103
66. Kondo S, Nakatsu S, Sakahara H, Kobayashi H, Konishi J, Namba Y (1993) Antitumour activity of an immunoconjugate composed of anti-human astrocytoma monoclonal antibody and neocarzinostatin. Eur J Cancer 3:420
67. Okamoto K, Yamaguchi T, Otsuji E, Yamaoka N, Yata Y, Tsuruta H, Kitamura K, Takahashi T (1998) Targeted chemotherapy in mice with peritoneally disseminated gastric cancer using monoclonal antibody-drug conjugate. Cancer Lett 122:231
68. Otsuji E, Yamaguchi T, Tsuruta H, Yata Y, Nishi H, Okamoto K, Taniguchi K, Kato M, Kotani T, Kitamura K, et al (1995) The effect of intravenous and intra-tumoural chemotherapy using a monoclonal antibody-drug conjugate in a xenograft model of pancreatic cancer. Eur J Surg Oncol 21:61
69. Makimoto H, Koizumi K, Tsunoda S, Wakai Y, Matsui J, Tsutsumi Y, Nakagawa S, Ohizumi I, Taniguchi K, Saito H, Utoguchi N, Ohsugi Y, Mayumi T (1999) Tumor vascular targeting using a tumor-tissue endothelium-specific monoclonal antibody as an effective strategy for cancer chemotherapy. Biochem Biophys Res Commun 260:346
70. Lode HN, Reisfeld RA, Handgretinger R, Nicolaou KC, Gaedicke G, Wrasidlo W (1998) Targeted therapy with a novel enediyene antibiotic calicheamicin θ^{I}_{1} effectively suppresses growth and dissemination of liver metastases in a syngeneic model of murine neuroblastoma. Cancer Res 58:2925
71. Sievers EL (2001) Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukaemia in first relapse. Expert Opin Biol Ther 1:893
72. Sievers EL, Appelbaum FR, Spielberger RT, Forman SJ, Flowers D, Smith FO, Shannon-Dorcy K, Berger MS, Bernstein ID (1999) Selective ablation of acute myeloid leukemia using antibody-targeted chemotherapy: a phase I study of an anti-CD33 calicheamicin immunoconjugate. Blood 93:3678
73. Hamann PR, Hinman LM, Beyer CF, Lindh D, Upeslacis J, Flowers DA, Bernstein I (2002) An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. Bioconjug Chem 13:40
74. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D, Mountain A, Flowers DA, Bernstein I (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconjug Chem 13:47
75. Rajvanshi P, Shulman HM, Sievers EL, McDonald GB (2002) Hepatic sinusoidal obstruction after gemtuzumab ozogamicin (Mylotarg) therapy. Blood 99:2310
76. van Der Velden VH, te Marvelde JG, Hoogeveen PG, Bernstein ID, Houtsmuller AB, Berger MS, and van Dongen JJ (2001) Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells. Blood. 97:3197
77. Knoll K, Wrasidlo W, Scherberich JE, Gaedicke G, Fischer P (2000) Targeted therapy of experimental renal cell carcinoma with a novel conjugate of monoclonal antibody 138H11 and calicheamicin theta-I1. Cancer Res 60:6089
78. Folkman J (1996) New perspectives in clinical oncology from angiogenesis research. Eur J Cancer 14:2534
79. Ran S, Gao B, Duffy S, Watkins L, Rote N, Thorpe PE (1998) Infarction of solid Hodgkin's tumors in mice by antibody-directed targeting of tissue factor to tumor vasculature. Cancer Res 58:4646
80. Matsuno F, Haruta Y, Kondo M, Tsai H, Barcos M, Seon BK (1999) Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies. Clin Cancer Res 5:371
81. Olson TA, Mohanraj D, Roy S, Ramakrishnan S (1997) Targeting the tumor vasculature: inhibition of tumor growth by a vascular endothelial growth factor-toxin conjugate. Int J Cancer 73:865
82. Arora N, Masood R, Zheng T, Cai J, Smith DL, Gill PS (1999) Vascular endothelial growth factor chimeric toxin is highly active against endothelial cells. Cancer Res 59:183
83. Arap W, Pasqualini R, Ruoslahti E (1998) Chemotherapy targeted to tumor vasculature. Curr Opin Oncol 10:560
84. Brekken RA, Huang X, King SW, Thorpe PE (1998) Vascular endothelial growth factor as a marker of tumor endothelium. Cancer Res 58:1952