

Bifunctional Antibodies: Preclinical and Clinical Applications

Casey W. Shuptrine and Louis M. Weiner

Abstract Treating malignancies with antibody-based immunotherapy has revolutionized the concept of targeted therapy. Rituximab and Trastuzumab, two monoclonal antibodies approved in the 1990s by the FDA, elucidated the potential of harnessing the immune system to eliminate transformed cells. As with any cancer therapy, a significant proportion of patients relapse, driving the development of non-traditional antibody-based therapies. Therefore, in an effort to enhance the ability of antibodies to retarget immune cells toward cancer cells, bispecific antibodies were born. Created through a variety of techniques they conform to an assortment of structures, recapitulating the basic structure of an antibody or deconvoluting the antigen-binding domains into unique designs. The European Union's approval in 2009 of Catumaxomab, a bispecific antibody that links cells of the innate and adaptive immune system to EpCAM+ cells for the treatment of malignant ascites, marks the first clinically approved dual-targeting antibody. Blinatumomab, a bispecific T-cell engager (BiTE), links T-cells directly to malignant cells, activating target-cell apoptosis through perforin-granzyme release. Early clinical results of Blinatumomab show a remarkable 80 % response rate in a heavily pretreated ALL patient subgroup. These enticing clinical results represent the forefront of the bispecific antibody field but evidence exists that point to the clinical success of numerous bispecific antibody formats. Although it is unknown which format will exhibit the highest clinical efficacy, it is clear that dual-targeting antibodies represent the future of immunotherapy for the treatment of cancer.

C.W. Shuptrine (✉)

Tumor Biology Training Program, Lombardi Comprehensive Cancer Center,
Georgetown University Medical Center, 3800 Reservoir Rd NW, Washington,
DC 20007, USA
e-mail: shuptrine128@gmail.com

L.M. Weiner

Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University
Medical Center, 3800 Reservoir Rd NW, Washington, DC 20007, USA

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1 Introduction

The clinical and commercial success of monoclonal antibodies has proven the hypothesis established by Paul Ehrlich in the 1900s that the immune system could provide a magic bullet for the treatment of cancer. While these single target antibodies have vastly improved survival for patients with HER2+ breast cancer, CD20+ lymphoma, and CD33 lymphoma, inevitably a significant proportion of patients relapse. With the proof of principle for immunotherapies, a revolution in the tumor immunology world began, with the goal of designing and developing techniques to overcome the current limitations of cancer targeting monoclonal antibodies (mAbs). This has led to the enhancement of effector functions through Fc region modifications, as well as the direct delivery of toxins to transformed cells through toxin-mAb conjugation (trastuzumab-DM1) [68]. A viable alternative method to enhance the efficacy of tumor immunotherapy is the design of antibodies or recombinant proteins that target multiple antigens and/or induce cancer cell destruction through the redirection of lytic immune cells. These dual-targeting antibodies are created through chemical conjugation, fusion of two mAb-producing hybridomas, and genetic recombination. The resulting bispecific antibody (bsAb) field has expanded immensely, with dozens currently undergoing various phases of clinical trials for the treatment of cancer and many more in preclinical studies. Of particular interest are trifunctional antibodies (Triomabs) developed by Trion Pharma and bispecific T-cell engagers (BiTEs) developed by Micromet. Other less clinically developed bsAbs include single-chain Fv (scFv) fusion proteins, diabodies, tribodies, bispecific CovX bodies, and random site mutation bsAbs. Although the structure and function of these bsAbs differ, they share antigen targets. On the tumor cell, they recognize members of the EGFR family, CD19, CD20, CD33, MCSPs, and EpCAM. For those that redirect effector cells, the antigen targets include CD3 on T-cells, CD16 on NK cells, monocytes, macrophages, and neutrophils, and CD64 on macrophages and monocytes. In this chapter, we describe the development of bispecific antibodies for the treatment of cancer in a historical perspective, highlighting the bsAbs that have entered clinical trials.

2 First Generation bsAbs

Before the development of advanced genetic recombination, bsAbs were created by the direct chemical crosslinking of antibody domains or through the fusion of two mAb producing hybridomas, called quadromas (Fig. 1) [56, 82]. Both methods

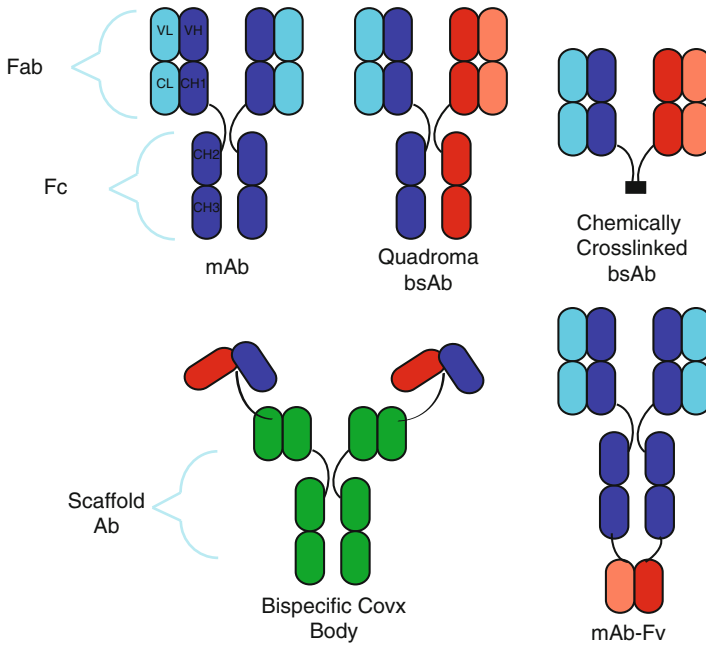


Fig. 1 First generation bispecific antibodies. *Dark regions* correspond to heavy chains, while *light regions* correspond to light chains. A monoclonal antibody is depicted in, with the Fab domain binding to an antigen and the Fc domain binding to FcγRs. *Red domains* represent regions with a different antigen specificity. The *green* antibody domain symbolizes the scaffold antibody for a bs-CovX body. *Black lines* represent linker domains. Bispecific antibodies of the quadroma, chemical crosslinking, bs-CovX body, and mAb-Fv formats are shown

produce a full-length antibody that can effectively bind to multiple antigens, either manipulating the downstream signals of the target or conjugating immune effector cells directly to the target cell. In the 1990s and early 2000s, several of these first generation bsAbs entered phase I clinical trials with limited success [26, 27, 92, 94, 116, 119]. For chemical crosslinking, the production of large quantities of purified bsAbs was cost prohibitive, as each reaction created numerous inert and unusable proteins. For the quadroma bsAbs, which were of murine or rat origin, the development of human anti-mouse antibodies (HAMA) or human anti-rat antibodies (HARA) precluded multiple dosing regimes in patients, severely limiting their clinical applicability. Also, quadromas created from the same species secrete ten possible combinations of light-chain and heavy-chain antibodies, with only one having the correct dual-targeting functionality [69]. A novel discovery was made in 1995 by Horst Lindhofer, that the fusion of a rat hybridoma with a murine hybridoma resulted in the preferential creation of the correct bsAb formation, increasing the yield from quadromas by 3.5-fold [69].

2.1 Chemical Conjugation

The first chemically cross-linked bsAb to enter clinical trials targeted HER2 and Fc γ RI (CD64), in an attempt to utilize macrophages and monocytes to lyse cancer cells. HER2 is one of the most targeted antigens by immunotherapy, as ~30 % of all breast cancers overexpress this potent tyrosine kinase receptor and is the target for the FDA approved mAb trastuzumab [11]. The murine bsAb, called MDX-210, was formed through the reduction of the two murine parental mAbs into Fab' fragments, chemically cross-linked using *o*-phenylenedimaleimide, acetylated with the alkylating agent iodoacetamide, and then purified through chromatography. Therefore, this molecule mimics the structure of a HER2 mAb, with two antigen-binding arms recognizing HER2 and one region recognizing an Fc γ R. The major difference compared to standard mAbs is the unique antigen-binding target of CD64, instead of Fc γ RIII (CD16), which IgG1 molecules recognize through their Fc region. MDX-210 was delivered intravenously to ten patients with HER2+ advanced breast or ovarian cancer, at a dose ranging from 0.35 to 10.0 mg/m². The bsAb was well tolerated in the ten evaluable patients, never reaching the maximum tolerated dose (MTD). Most patients only experienced grade 1 and 2 adverse events but two experienced the grade 3 adverse event hypotension. Although the goal was only to test the toxicity of the molecule, MDX-210 treatment resulted in one partial and one mixed tumor response. As expected, HAMA was detectable in six of the ten patients [116]. This observed limiting factor prompted the development of a humanized version of the bsAb, MDX-H210, which was tested in patients with metastatic HER2+ breast cancer in two phase I trials. To enhance the proportion of immune cells with lytic capabilities, these investigators then coadministered MDX-H210 with granulocyte-colony-stimulating factor (G-CSF), which has been shown to induce the expression of CD64 on neutrophils. In the first clinical trial, of the 23 patients enrolled, the majority of patients experienced fevers or diarrhea, while no MTD was reached. No objective clinical responses were observed and all patients had circulating antibodies that recognized the bsAb [92]. The second phase I trial of MDX-H210 was delivered to 30 stage IV HER2+ breast cancer patients in 2003. Similar to the previous trials, no MTD was reached, the molecule was well tolerated, no objective responses were seen, and a majority of patients developed anti-bsAb antibodies [94]. Another chemically conjugated bsAb, MDX-447, which targets EGFR and CD64, was evaluated with and without G-CSF in 64 patients with advanced solid tumors. Although the addition of G-CSF caused dose-limiting toxicities, MDX-447 was relatively tolerated and resulted in no objective clinical responses, halting its further development [34].

The lack of clinical activity in the patients treated with these conjugated molecules might be explained by the strong pre-stimulation of polymorph nuclear neutrophils (PMN) cells with IFN- γ and G-CSF, and the high effector to target ratio of PMNs to tumors cells (50–200:1) that is required for *in vitro* lysis by MDX-210 and MDX-H210 [57]. Moreover, the development of neutralizing antibodies or the lower lytic capability of CD64 expressing immune cells might have attributed to the lack of clinical efficacy.

2.2 *Quadromas*

The first generation of quadroma bsAbs recognized CD16 or CD3 to link lytic immune cells directly to tumor cells. The resulting bsAbs use one Fab arm to recognize the tumor antigen, another Fab to bind to CD16 or CD3, and use an intact Fc domain to bind to CD16 and FcRn. Therefore, CD16×tumor antigen bsAbs have two unique binding domains for CD16 on innate immune cells, theoretically enhancing their ability to mediate antibody-dependent cellular cytotoxicity (ADCC). This design was utilized for two murine bsAbs that entered phase I clinical trials in the 1990s, 2B1 and HRS-3/A9. 2B1, which targets HER2/neu and CD16, was utilized in two clinical trials for patients with HER2+ tumors. Preclinical data suggested that the 2B1 bsAb derived from the 520C9 and 3G8 fusion quadroma was effective in eliminating HER2+ target cells *in vitro* and potentiating their growth *in vivo* [117, 118]. Similar to MDX-210, the effector cells required significant stimulation and high E:T ratios (25–50:1). Minimal clinical activity was observed following 2B1 therapy, with a total of 33 out of 34 patients developing HAMA [15, 119]. Of particular interest was the induction of an adaptive immune response to HER2/neu, suggesting that 2B1 enhanced antigen presentation for HER2. However, this adaptive immunity did not translate to clinical responses. HRS-3/A9 was created through the fusion of the murine hybridomas HRS-3, which produces a mAb for CD30, and A9, which produces a mAb against CD16 [52]. CD30, a marker for Hodgkin lymphomas, represents a valid target for selective targeting of lymphoma cells [111]. Preclinical studies with HRS-3/A9 were promising, as it was shown to cure mice with CD30+ Hodgkin's lymphomas after only one injection [52]. In 1997, HRS-3/A9 was given to 15 patients with refractory Hodgkin's disease, was well tolerated, and resulted in the first clinical responses seen with a bispecific antibody, with one complete response (CR), one partial response (PR), and three minor responses [43]. Similar results were seen in a second phase I trial, with one CR, three PRs, and four patients with stable disease (SD) [44]. While these results elucidated the potential of bsAbs for the treatment of cancer, 15 of 31 patients developed HAMAs, prohibiting subsequent clinical use [43, 44].

In an effort to enhance the effector functions of antibody therapy, many groups sought to link the most powerful arm of the adaptive immune system to cancer cells. By conjugating CTLs through CD3, directly to a tumor cell through a tumor-associated or -specific antigen, effective lysis can occur. After being primed in the lymph nodes through ligation of CD3 and a second activating signal, CTLs disseminate throughout the body. Through the T-cell receptor (TCR), educated CTLs recognize their “primed antigen” presented on the surface of a cell through major histocompatibility complex (MHC) class I. Upon conjugation, CTLs release perforin and granzymes, activating the apoptotic pathway inside the target cell [81]. Therefore, the creation of a second antigen-binding domain of an antibody to recognize the activating T-cell receptor CD3 could effectively utilize the cytotoxic arm of the adaptive immune system to eliminate cancer cells. Unfortunately, when given to patients, T-cell retargeting bsAbs resulted in a systemic cytokine release induced by

T-cell activation. This, on top of the development of HAMA, significantly limited the bsAb dose, reducing their clinical efficacy.

The first T-cell retargeting quadroma was SHR-1, a rat/murine hybrid that recognized CD3 and the B-cell lymphoma marker, CD19. Preclinical studies found that SHR-1 could lyse CD19+ B-cell lines and B-cells taken directly from lymphoma patients with activated T-cells [39, 40]. SHR-1 was capable of mediating significant lysis of transformed B-cells with T-cells isolated from patients with lymphoma, proving that CD3 could serve as a potent activator of previously tolerant T-cells. Further in vitro studies demonstrated complete cell growth inhibition at a relatively low E:T ratio of 9:1 when SHR-1 was combined with IL-2 stimulated peripheral blood mononuclear cells (PMBCs) [41]. These promising results led to a phase I clinical trial in 1995, where three non-Hodgkin's lymphoma (NHL) patients were exposed to increasing doses of SHR-1. Although the bsAb was well tolerated with little observed toxicity, clinical activity was minimal [26, 27]. HEA125×OKT3, another first generation quadroma bsAb of murine origin, retargeted T-cells to EpCAM (CD326) positive tumors. EpCAM expression is commonly associated with poor prognosis, is expressed on a broad range of carcinomas, and is often used as a marker for stem cell-like properties [83, 86, 109]. EpCAM is also expressed on normal cells but is generally confined to intracellular spaces, making it an attractive target for anticancer therapy. In 2002 HEA125×OKT3 was evaluated in ten EpCAM+ ovarian carcinoma patients with malignant ascites. At the time, patients with malignant ascites, which are tumor cells that metastasize to the peritoneal cavity causing pain and swelling, had limited options for palliative treatment. Diuretics or direct fluid drainage through paracentesis were the only viable options to improve quality of life. Since the malignant epithelial cells in ascites caused by ovarian carcinoma are often EpCAM +, treatment with HEA125×OKT3 was considered to potentially fill a significant unmet need in cancer palliative care. When injected intraperitoneally, HEA125×OKT3 resulted in the complete inhibition of ascites formation in eight patients and reduced ascites formation in two patients. However, 80 % of the patients developed HAMAs, seemingly slowing its further clinical development [77].

2.3 *Triomabs*

2.3.1 **Early Generation Triomabs**

Triomabs, the most successful bsAb created using the quadroma technique, are the only type of bispecific antibody used to treat cancer. Since first generation quadromas produced ten antibody formations randomly, it was difficult to purify the correct dual-targeting antibody. Utilizing the discovery of preferential pairing of bsAbs in mouse IgG2a/rat IgG2b quadromas and a single step pH elution on protein A, the desired bsAb could be isolated with previously unimagined ease [69]. Surprisingly, the Fc region of these triomabs bound only to activating FcγRs (Fcγ RI and Fcγ RIII) on effector cells and not to inhibitory receptors (Fcγ RIIB) [123]. This prompted the development of numerous commercially viable bsAbs, targeting EpCAM, HER2,

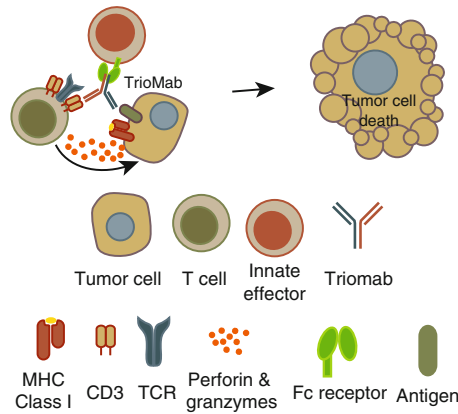


Fig. 2 Triomab’s mechanism of action. Trifunctional antibodies, such as catumaxomab, ertumaxomab, and Bi20, lyse tumor cells by retargeting T-cells to antigen expressing tumor cells. The Fc region of the triomab binds to cells of the innate immune system, such as NK cells, which can induce ADCC of the tumor cell as well as supplying stimulatory signals to the retargeted T-cells. Once conjugated, T-cells or NK cells release perforin and granzymes to induce apoptosis of the tumor cell

CD20, melanoma-associated proteoglycans, and the melanoma-associated gangliosides GD2 and GD3, all of which utilize CD3 as their secondary binding target [70]. The first proof of principle triomab targeted against EpCAM, called BiUII, could mediate lysis of PCL-1 and FaDu, squamous carcinoma cells of the head and neck, with unstimulated PBMCs [122]. This was evident at relatively low concentrations of BiUII (5 ng/ml) and could mediate higher lysis of target cells than a combination of both parental CD3 and EpCAM antibodies. Therefore, the direct linkage of T-cells, NK cells, and tumor cells appears to impart a cytotoxic advantage, possibly due to the secondary activation of T-cells by NK cells or macrophages (Fig. 2). Although a high E:T ratio of 20:1 was required to reach specific lysis near 90 %, BiUII was capable of mediating 60 % and 25 % lysis at the conservative E:T ratios of 5:1 and 1:1, respectively [122]. Other preclinical EpCAM triomabs were shown to mediate significant lysis of EpCAM positive prostate cancer cell lines as well as protect 100 % of the mice from secondary challenge with B16 melanoma cells 144 days after initial inoculation [96, 98]. The induction of an adaptive immune response was likely due to the triomab’s ability to activate dendritic cells, T-cells, and macrophages [123]. These trifunctional antibodies overcame the limitations of large-scale purification and were able to link the innate and adaptive together in a common anticancer goal, prompting their clinical development.

2.3.2 Catumaxomab (Removab)

Learning from the design of previous EpCAM triomabs, catumaxomab (Removab®) was developed specifically for the clinic and similar to HEA125×OKT3, was

utilized for the treatment of malignant ascites. Initial phase I results demonstrated limited toxicity of catumaxomab therapy when delivered intraperitoneally to seven patients in 2005, prompting a phase I/II trial with 23 women presenting with malignant ascites [46]. Patients received 4–5 regimens of catumaxomab at increasing doses starting from 10 μg and ending at 200 μg . Although a significant proportion of patients experienced grade 3 adverse events, the majority of events were reversible. Remarkably after the 37 day trial, only one of the twenty-three patients required paracentesis, a significant improvement from the median time to paracentesis of 7–11 days commonly seen in this patient subgroup [21]. After the final treatment, those treated with catumaxomab saw a 99.9% reduction in the mean EpCAM+ tumor cell numbers in the peritoneal fluid, clearly demonstrating the antitumor ability of catumaxomab [21]. Using the dose established in this trial, a second phase II trial was conducted for 13 patients with malignant ascites to determine the pharmacokinetics and efficacy of catumaxomab. At days 0, 3, 6 or 7, and 10, patients received doses of 10, 20, 50, and 150 μg of the triomab intraperitoneally. The half-life was determined to be 2.13 days and although peritoneal concentrations were consistently high ranging from 552 to 6,121 $\mu\text{g}/\text{ml}$, the systemic levels in the serum remained extremely low, with a maximum concentration of 403 $\mu\text{g}/\text{ml}$ [100]. While the low systemic concentration could limit the efficacy of Catumaxomab, it likely reduced the toxicity of this therapy. After a single 10 μg dose of Catumaxomab, the median EpCAM+ tumor cell count reduced from 9,362 tumor cells per million ascites cells to only 49 [100].

A landmark phase II/III trial (NCT 00836654) with 258 patients with recurrent symptomatic malignant ascites caused by ovarian or non-ovarian cancer proved that intraperitoneal treatment of catumaxomab leads to a significant enhancement in puncture-free survival, median time to next paracentesis, and higher overall survival in gastric cancer patients. Utilizing the same increasing dose regimen as the previous phase II trial, patients were separated into two groups: paracentesis and catumaxomab (treatment) or paracentesis alone (control). Catumaxomab's addition resulted in an increase in puncture-free survival from 11 days in the control group to 46 days and enhanced the median time to paracentesis from 13 days to 77 days [47]. Catumaxomab also completely eliminated the EpCAM+ tumor cell count in the ascites fluid in 95 of the 115 evaluable samples. The adverse events associated with catumaxomab were widespread, affecting 98% of the patients. Toxicities from cytokine release were well controlled and reversible. Eight days after the last infusion, 76% of all patients (85/112) presented with HAMA even though only 5% (6/124) of patients had detectable HAMAs before the last infusion of 150 μg [47]. In a post hoc analysis, the induction of HAMA did not adversely influence clinical benefits, but instead suggested that the development of HAMA could effectively be used as a biomarker for responders to catumaxomab treatment [89]. A second post hoc analysis demonstrated that peritoneal samples from catumaxomab-treated patients had significantly lower tumor cell counts, no stem cell-like tumor cells (CD133+/EpCAM+), lower VEGF levels, and double the number of activated CD4+ and CD8+ T-cells compared to the control group [54]. Early results of this Phase II/III trial prompted the European Union to approve the use of catumaxomab

for the treatment of malignant ascites in 2009, as no effective therapeutic existed for this patient group. Catumaxomab is the first and only bispecific antibody to receive approval for clinical use as a cancer therapeutic.

However, as EpCAM is expressed on such a broad range of carcinomas, it could be utilized for the treatment of numerous different cancers. As the most similar indication to malignant ascites, catumaxomab has been used in a phase II trial to treat patients with malignant pleural effusions caused by breast cancer or NSCLC. Three escalating intra-pleural doses of 5–200 µg in the 24 patients led to significant adverse events, with two dose limiting toxicities (DLTs). Although the trial was stopped early due to the high number of serious adverse events (23) and dropouts, one CR and four PRs associated with pleural effusion, occurred in the breast cancer group. Not surprisingly, most patients developed HAMA [107]. Since catumaxomab has the potential to eliminate solid tumors, one phase I and one phase II trial have been performed for NSCLC and epithelial ovarian cancer, respectively. The phase I trial represents the only instance where catumaxomab was delivered intravenously to patients. Utilizing an increasing dosing regimen of 2–7.5 µg, the maximal tolerable dose was determined to be 5 µg when administered with 40 mg of dexamethasone, a potent anti-inflammatory agent [106]. Surprisingly, no patients developed HAMA or HARA when exposed to catumaxomab, although one patient, who had preexisting HARA before treatment, had elevated levels by the end of the trial. In an effort to directly treat patients with epithelial ovarian cancer, catumaxomab was delivered intraperitoneally to 45 patients in 2011. Stratified into two groups, the low dose group of four infusions of 10 µg resulted in two patients with stable disease. The high dose group received infusions of 10, 20, 50, and 100 µg of catumaxomab, leading to one partial response and five patients with stable disease. Every patient exhibited a treatment-related adverse event, 17 of 45 patients developed HAMA or HARA, and no difference in progression-free survival was seen between the two groups [12]. Additional trials are being performed to determine the efficacy of catumaxomab as an anticancer therapeutic for indications different from malignant ascites. As of 2012, catumaxomab is being utilized in close to a dozen clinical trials with the most notable being a phase III trial for malignant ascites with or without the anti-inflammatory drug prednisolone (NCT 00822809).

2.3.3 Ertumaxomab (Rexomum)

The second triomab, ertumaxomab, was developed with the same CD3 binding arm of catumaxomab, to retarget T-cells and innate killer cells to HER2+ tumor cells. When compared to trastuzumab at a high E:T ratio of 20:1, ertumaxomab-mediated maximum lysis of high HER2 expressing cells, classified as HER2 3+, at a fivefold lower concentration (1 ng/ml vs. 5 ng/ml). Reducing the E:T ratio to 5:1 to closer reflect in vivo conditions, trastuzumab only mediated 40 % lysis of tumor cells at maximum concentrations of 1–5 µg, while ertumaxomab was able to mediate total tumor lysis at 25 ng/ml [53]. One major limitation of trastuzumab therapy is its inability to lyse tumor cells that express low levels of HER2. In the same preclinical

study, trastuzumab only lysed 10 % of 1+ HER2 cells at a concentration of 5 $\mu\text{g/ml}$, while ertumaxomab mediated 100 % tumor cell killing at 5 ng/ml , at an E:T ratio of 20:1. This potency was maintained when the effector cells were significantly reduced [53]. Therefore, ertumaxomab mediates significantly improved lysis of HER2+ cancer cells at low E:T ratios and can promote the lysis of cells that are typically resistant to trastuzumab-promoted ADCC. As of 2012, ertumaxomab has been studied in two phase I clinical trials, in order to determine its toxicity profile. The first phase I trial, with four malignant ascites patients, demonstrated that ertumaxomab was well tolerated, resulting mainly in flu-like symptoms, including fever, chills, and fatigue [46]. This prompted a second phase I trial for 15 patients with HER2 expressing metastatic breast cancer. Utilizing a similar dosing regimen as catumaxomab, ertumaxomab was administered i.v. on days 1.7 ± 1 and 13 ± 1 with an initial dose of 10 μg and a final dose of 200 μg . Again, most adverse events were associated with flu-like symptoms. A few adverse events were considered serious, based on grade 3 and 4 lymphocytopenia and an increase in liver enzymes. All adverse events were reversible and the MTD was determined to be 100 μg . Of the 15 evaluable patients, one patient experienced a CR, two had PR, and two had SD [59]. Measurement of cytokine levels revealed that ertumaxomab elicited a strong Th1 cytokine response, as IL-6, IL-2, TNF- α , and IFN- γ were all elevated. Also, surprisingly, only five of the fifteen patients developed HAMA or HARA. Currently, ertumaxomab is being studied in one phase I/II clinical trial for the treatment of HER2+ solid tumors (NCT 01569412).

2.3.4 Bi20 (FBTA20)

A third Triomab, Bi20 or FBTA20, targets CD20 on lymphoma cells, linking T-cells and innate immune cells through the same CD3 domain and the intact Fc domain, respectively, as the other two triomabs. Preclinical data demonstrated the potent ability of Bi20 to mediate cytotoxicity of CD20+ B-cell lines at E:T ratios of 5:1. At a concentration of 50 ng/ml , Bi20 killed 95–100 % of B-cells, while rituximab, the FDA approved mAb to treat CD20 cancers, only mediated 65 % of B-cell lysis at 50 $\mu\text{g/ml}$ [110]. In contrast to rituximab, Bi20 mediated enhanced T-cell and monocyte/macrophage activation and proliferation, a strong Th1 cytokine response, and lysed low expressing CD20 B-cells. These findings were replicated using B-cells isolated from patients with chronic lymphocytic leukemia, where Bi20 mediated efficient lysis even when CD20 expression was extremely low [14]. A phase I trial of i.v. delivered Bi20 for CLL and NHL patients in combination with donor lymphocytes, demonstrated mild adverse events of fever, chills, and bone pain. None of the six patients developed HAMA with Bi20 treatment and survival ranged from 38 to 486 days [20]. Bi20 is currently not being tested in any clinical trials but could prove to be efficacious in B-cell malignancies.

Also of interest are two triomabs created in 2004 that target melanoma-associated proteoglycans (TRBs02) and the melanoma-associated gangliosides GD2 and GD3 (TRBs07). The parental hybridomas B5 and Me361 were each fused

with the CD3 hybridoma 26H6 to create TRBs02 and TRBs07, respectively. Both Triomabs were capable of inducing a Th1 cytokine response when exposed to tumor cells and PBMCs, associated with TNF- α , IL-2, IL-6, and IFN- γ , as well as the immunosuppressive cytokines IL-10. TRBs02 and TRBs07 induced the proliferation of CD8+ and CD4+ T-cells but reduced the number of NK cells and monocytes [99]. Lysis of antigen presenting cells was modest compared to other triomabs but combining both bsAbs was especially potent against cells expressing both tumor antigen targets.

2.4 *Alternative Full-Length BsAbs*

With the first clinical approval of a bsAb as well as extremely promising preclinical and clinical results of other bsAbs, there has been a renewed interest in developing methods to produce non-immunogenic, production-scalable bsAbs. These include two novel redox reactions, a knobs-into-holes method, a CDR mutational method, bispecific CovX bodies, and a mAb with an extra Fv domain. Both redox reactions take existing mAbs and expose them to either reducing agent, 2-mercaptoethane-sulfonic acid sodium salt (MESNA) or glutathione (GSH), respectively [22, 112]. These methods result in the relatively quick formation of a bsAb with one binding domain for each antigen. Strop and colleagues mutated the hinge region with oppositely charged amino acids and the CH3 domain of each separately expressed mAb. This method facilitates the stabilization of the bispecific antibody format. As a proof of principle, using this method, they created a HER2 \times EGFR and a CD20 \times CD3 bsAb, which have hybrid IgG1/IgG2 Fc domains [112]. The CD20/CD3 bsAb mediated 80 % lysis of the CD20 positive cell line, A20, at 1 μ g/ml with a low E:T ratio of 5:1 and could mediate a depletion of CD19+ A20 cells in vivo. The proof of principle bsAbs created from Heath's group demonstrated that the redox method could create bsAbs in 6–10 days of same species or cross species fusions [22].

Another unique method utilizes the knobs-into-holes technology originally hypothesized by Watson and Crick and developed in the 1990s. By replacing a small amino acid (AA) side-chain with a large AA side-chain, or “knob,” in the CH3 domain of one antibody and replacing a large AA side-chain with a small AA side-chain, or “hole,” in the CH3 domain of another antibody, the yield of bsAbs can be significantly increased [95]. This discovery enhances the heterodimerization of dual-targeting antibodies and reduces the different types of possible isomers to four. As one example, they created a bsAb targeted against VEGF-A and Ang-2, showing that the bsAb bound to both antigens with the same affinity as the parental mAbs. This bsAb also inhibited in vivo growth of Colo205 cells by 92 %, while the combination of both mAbs were somewhat less efficient, inhibiting growth by 78 % [101, 102]. Therefore, targeting two growth factors with one antibody may impart an enhanced antitumor effect.

One group, in an effort to approach the bi-specificity from a different angle, developed an intriguing method that results in two different antigen-binding

domains on each Fab arm. Starting with a full-length mAb, this group creates a library of variants with mutated light-chain (LC) complementarity determining regions (CDRs). All variants are then analyzed for binding to the two antigens of interest, with the goal of isolating a variant with strong affinity for two targets. The first of these “two-in-one” bsAbs was created from trastuzumab and mutated to also bind to VEGF. The best dual-targeting variant bH1 underwent a second round of mutations to enhance the affinity for each antigen. The resulting high affinity variant, hB1-44, was able to inhibit the growth of Colo205 cells *in vivo* better than either parental antibody alone [16]. Another two-in-one antibody, MEHD7945A, was created using a phage display library of mutated Fab domains to target HER3 and EGFR. As before, this bsAb has identical Fab arms and is capable of binding either antigen with high affinity. MEHD7945A was capable of inactivating the EGFR and ERK pathway at an $IC_{50}=0.03$ and $0.16 \mu\text{g/ml}$, respectively, inhibiting growth of EGFR+ lines *in vitro*, and stunting the growth of EGFR+/HER3+ cells *in vivo*. This was compared to the combination of the two parental mAbs [101, 102]. Also, this bsAb could mediate ADCC and when tested in cynomolgous monkeys, elicited significantly less dermatological toxicity than the FDA approved EGFR parental antibody, cetuximab. The clinical dose was determined to be 8–12 mg/kg of MEHD7945A and is currently being evaluated in a phase I trial for patients with epithelial tumors (NCT01577173) [55].

Other promising chemical conjugation techniques are bispecific CovX bodies and mAb-Fv fusions. The bispecific CovX body is a unique method that utilizes the same Fc backbone, a so-called scaffold antibody CVX-2000, with interchangeable antigen-binding domains, allowing for rapid creation of mAbs or bsAbs. The antigen binding domains are chemically linked using an azetidione linker to the scaffold antibody after the antigen peptides are fused together using maleimidethiol ligation. The first of these bs-CovX bodies, CVX-241 targeted Ang-2 and VEGF-A, with bivalent binding on each Fab arm, as each arm of the antibody can bind to either antigen [30]. CVX-241 was capable of inhibiting the growth of Colo205 cells in a xenograft model, compared to the combination of Ang-2 and VEGF-A monoclonal CovX bodies. Of particular interest, the bispecific antibody synergized with the common chemotherapeutic agent irinotecan to significantly inhibit *in vivo* growth. The first phase I trial with CVX-241 was terminated due to poor pharmacological properties at the highest dose of 25 mg/kg (NCT 01004822). Instead of trying to manipulate the basic binding structure of mAbs to allow for a second binding site, another method fuses an Fv region to the Fc domain of a mAb. If the Fv domain targets CD3, then the resulting bispecific antibody, called a mAb-Fv, can mediate antigen binding, ADCC, and retargeting of T-cells, similar to triomabs. The first set of these antibodies targeted $HER2 \times CD3 \times CD16$ and $HM 1.24 \times CD3 \times CD16$. Surprisingly the HER2 mAb-Fv variant bound to CD16 with higher affinity and mediated greater than sevenfold higher lysis via ADCC than the parental mAb trastuzumab [85]. Moreover, the mAb-Fvs still bound to FcRn with similar affinity as the parental antibodies, suggesting that serum half-life of the fusion antibodies will not be compromised.

3 Recombinant Bispecific Antibodies

3.1 Tandem scFv

As an alternative approach to creating full-length bsAbs, many groups have chosen to manipulate the inherent structure of the antibody itself, creating a plethora of unique molecules. The basic design of these recombinant proteins involves deconvoluting the structure of an antibody down to the elements that are vital for antigen recognition and binding. With two binding sites for antigens and no Fc domain, these proteins retarget immune cells to tumor cells through CD3 or CD16. Utilizing T-cells or innate killer cells, they can mediate significant lysis of antigen expressing cells (Fig. 3). Expressed in bacterial or mammalian cells, the recombinant proteins are purified using a variety of techniques. The first developed technique fused two single-chain variable regions (scFvs) together using a peptide linker usually between 15 and 20 AA in length, creating a tandem scFv (TaFv). The AA linker length imparted flexibility for the scFvs, allowing for the correct domains to form together. Early designs demonstrated the lytic capability of these immune cell retargeting antibodies, as they were more cytotoxic than their parental mAbs [28, 37]. One TaFv, developed in an effort to circumvent the lack of success seen by the quadroma 2B1, targeted HER2 and CD16. The protein-mediated significant lysis of HER2 overexpressing cells and demonstrated good tumor retention in vivo [79]. rM28, a unique TaFv, targeted the melanoma-associated proteoglycan (NG2) and redirected T-cells through CD28 activation. Therefore, rM28 links tumor cells to T-cells while

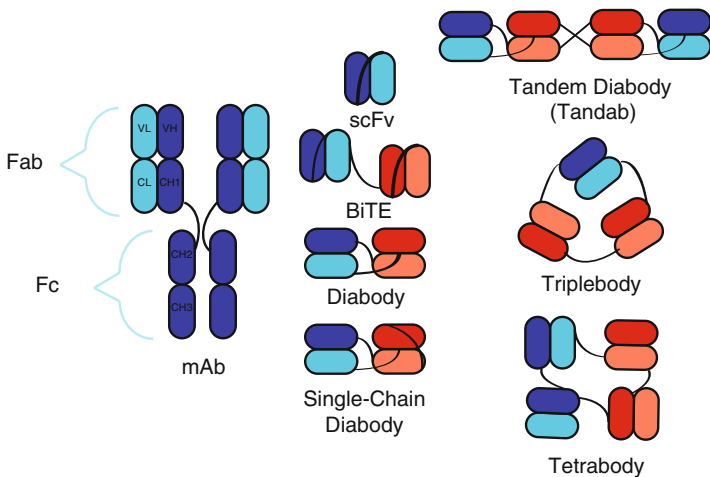


Fig. 3 Recombinant bsAbs. The antigen binding components of a mAb are isolated to create smaller antibodies, reducing their stability and immunogenicity. *Black lines* represent linker domains. Bispecific antibodies of the diabody, single-chain diabody, BiTE, tandab, triplebody, and tetrabody formats are shown

simultaneously providing them with a potent activating signal, resulting in “targeted supra-agonistic CD28 stimulation” directly at the tumor site [36]. Exciting preclinical results moved this bsAb to a phase I/II clinical trial in 2005 (NCT 00204594). Unfortunately, this trial was terminated due to the significant adverse events seen in a phase I trial of the CD28 mAb TGN1412, which caused life-threatening cytokine storm in treated volunteers [113]. A successor TaFv that targets CD20 and CD28 was then created using the same CD28 scFv. r2820 eliminated CD20+ lymphoma cells at modest antibody concentrations (0.5 $\mu\text{g/ml}$) [90]. E3Bi, another TaFv created using a 14 AA linker sequence, targeted EpCAM and CD3. This molecule was capable of eliminating EpCAM+ cells both in vitro at extremely low E:T ratios of 1–2.5:1, and in vivo, where it significantly inhibited tumor growth [93]. An alternative approach to the retargeting of immune cells is the inhibition of growth factor receptor signaling. One example of this method is the scFv fusion protein, MM-111, which recognizes HER2 and HER3, inhibiting the intracellular signaling of both tyrosine kinase receptors [80]. This fusion protein differs in the basic structure from other TaFvs, as it is also bound to modified human serum albumin, in an effort to enhance MM-111 serum stability. Further studies need to be done to determine if this type of scFv fusion has antitumor capabilities, as it lacks any effector cell-mediated lytic potential. It is currently involved in three phase I clinical trials for HER2-amplified cancers.

3.2 Diabody

Diabodies (Db) are another recombinant bsAb that have been intensively studied. These proteins consist of the same scFv domains as TaFvs but utilize a shorter linker sequence. This results in a reduced yield of homodimers, essentially driving the formation of the bispecific format. Specifically, the heavy-chain variable domain (VH) and the light-chain variable domain (VL) of each antigen binding chain are forced to connect with respective domains on the second antigen chain. In other words, for antigens A and B, TaFvs read VH(A)—VL(A) (linker) VH(B)—VL(B), while Dbs read VH(A)—VL(B) (linker) VH(B)—VL(A). This allows for bivalent antigen binding on each half of the Db. As of 2012, over 40 different types of bispecific Dbs have been developed in preclinical studies, but none have entered human trials. This is mainly due to the short half-life seen with these small molecules, as continuous infusion appears to be necessary for adequate serum concentrations. The first Db design targeted the hapten phenyloxazone and hen egg lysozyme, was expressed in *Escherichia coli* (*E. coli*), and purified using affinity chromatography, yielding 0.3–1 mg/L [49]. The first Db to be tested in vivo targeted HER2, was almost cleared from the blood within 4 h but was retained in the tumor for close to a day [1]. Holliger and colleagues also developed the first Db that retargeted T-cells to tumor cells, using CD3 to link T-cells to BCL-1 + lymphoma cells. At a molecular weight of 50 kDa, this Db was capable of mediating 80 % lysis of lymphoma cells at the extremely low concentration of 63 ng/ml [50]. It also induced significantly

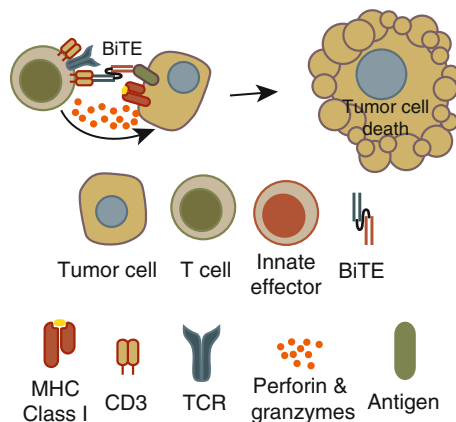
higher lysis than a CD3×BCL-1 quadroma bsAb, showing for the first time that the Db format could mediate higher tumor lysis than a similar quadroma bsAb.

Of the CD3 T-cell retargeting Dbs CD19, CEA, EGFR, Endoglin, and PSMA Dbs are of particular interest. All five of these Dbs were expressed in *E. coli*, with a yield ranging from 0.1 to 3 mg/L [33, 45, 51, 60, 65]. The CD19×CD3 Db-mediated lysis of CD19+ lymphoma cells at a tenfold lower concentration than a CD19×CD3 quadroma bsAb, although the E:T ratios and maximum lysis were modest [60]. Targeting CEA expressing colon carcinoma cells with IL-2 stimulated PBMCs and 100 ng/ml of the respective Db, resulted in 50 % lysis at the moderate E:T ratio of 10:1 [51]. The EGFR Db, Ex3, only mediated 60 % lysis at 1 µg/ml with 100 times more lymphokine-activated killer cells than EGFR expressing bile duct carcinoma cells [45]. Therefore, the group attempted to enhance the lytic capacity by humanizing the diabody, creating hEx3. This Db was able to mediate 100 % tumor inhibition at 0.5 pmol/ml at an E:T of 5:1 and enhanced in vivo survival from 10 weeks (control) to 23 weeks [6]. The endoglin targeting Db-mediated 50–60 % maximum lysis of endothelial cells at high LAK levels [65]. Lastly, the PSMA single-chain Db (scDb), which conforms to a slightly different shape with the binding domain for PSMA and CD3 existing on opposite sides of the molecule, was able to significantly inhibit tumor formation of prostate carcinoma cells in mice [33]. The remaining immune retargeting Dbs bind to CD16 and CD19, CD30, or EGFR. Not surprisingly, these Dbs required significantly high E:T ratios to mediate effective lysis of target cells [5, 8, 62].

3.3 Multi-Targeting Antibodies

In an effort to enhance the serum stability of these recombinant proteins, increase the avidity for an antigen, or increase the number of targets, groups added additional antigen binding domains. Similar to the CD3 x CD19 Db, Kipriyanov et al. created a Tandab, or tandem diabody, that targeted the same antigens. This format results in a protein that's double the size of Dbs (~114 kDa), increasing half-life, with four Vh–Vl domains instead of two [61]. This Tandab was able to stimulate T-cells, elicit higher lysis, and significantly inhibit the growth of in vivo lymphoma cells compared to the CD19×CD3 Db [24, 61]. Another group developed a similar molecule, called a tetrabody, from the humanized Ex3 Db. Although this molecule has four binding domains, similar to Tandabs, it is arranged in a circular format instead of a linear one. This hEx3 tetrabody was 113 kDa and could mediate the same lysis as the Db but at a 100-fold lower concentration, resulting in an extremely low EC50 of 0.5 fmol/ml at an E:T of 5:1 with LAK cells [7]. The triplebody, another bsAb format which binds to three antigens, is created by linking three scFvs together. Four promising candidates, CD19×CD16×CD19, CD33×CD123×CD16, CD123×CD16×CD123, CD33×CD19×CD16, exhibited enhanced stability, were expressed in mammalian cells, and induced half-maximum lysis in the low picomolar range [58, 67, 105]. As these molecules have the potential to form aggregates when in suspension, it remains to be seen if their potent lytic capabilities will translate safely into humans.

Fig. 4 BiTE's mechanism of action. Bispecific T-cell engager's function by linking T-cells directly to tumor cells through CD3 on T-cells. Conjugation of T-cells to tumor cells results in the release of perforin and granzyme, inducing apoptosis in the target cells. BiTEs exist that target CD19, EpCAM, EGFR, CD33, MCSPs, CEA, and EphA2



3.4 Bispecific T-Cell Engagers

3.4.1 Early Generation BiTEs

Even though BiTEs, or bispecific T-cell engagers, have not been evaluated in phase III clinical trials, they may represent the future of the bsAb field. The concentrations required to induce significant lysis *in vitro* and *in vivo* are some of the lowest seen for any anticancer therapy. By redirecting T-cells to antigen positive tumor cells, this approach is not unique. What is unique, is the ability to eliminate cancer cells at E:T ratios that are orders of magnitude lower than other bsAbs (1:1 or 1:10) with PBMCs that do not require pre-stimulation with IL-2 or CD28 activation [66, 121]. As two scFvs bound together with a short gly₄-ser₁ linker, the bispecific molecules are only approximately 55 kDa and are capable of binding only one antigen on each arm (Fig. 4). Currently, Micromet is in various stages of preclinical and clinical tests involving ten BiTEs that target eight different antigens. They are CD19, EpCAM, HER2/neu, EGFR, CEA, CD33, EphA2, and MCSPs [9]. One of the most surprising features of these bispecific molecules is their ability to mediate potent lysis without the need for MHC Class I [87]. Therefore, these molecules can utilize T-cells to target any type of cell, regardless of the T-cell's ability to inherently recognize the target cell. Importantly, BiTEs address previous toxicity limitations seen with earlier CD3-based bsAbs by utilizing a lower binding affinity to CD3, allowing for T-cell activation only at tumor sites. Therefore, BiTEs lead to specific antitumor effects without potentially life-threatening host toxicity caused by excessive cytokine release.

MT103 and MT110, which target CD19 and EpCAM, respectively, are the only two BiTEs in clinical trials but many more will follow soon, as all published results for these molecules are promising. The EpCAM-targeted BiTE is only undergoing a phase I clinical trial, when it was the first developed. In 1995, Mack et al.

developed the scFv fusion protein targeting CD3 and EpCAM. This molecule was capable of only being expressed in CHO cells, as *E. coli* expression resulted in inert protein [75]. Fortunately, the harvest of the EpCAM scFv fusion was significantly higher than other recombinant bsAbs, yielding 12–15 mg of protein per liter of media. This early generation BiTE was capable of inducing significant lysis at only 8 ng/ml and was stable in PBS for 6 months and serum for 56 h [66, 75]. Not surprisingly, CD8+ and CD4+ T-cells were required for the antitumor lysis [66]. The early generation EpCAM BiTEs could induce substantial lysis at concentrations near or below 1 ng/ml, all utilizing unstimulated PBMCs from healthy and cancer patients [76, 103, 120]. One in vivo study found that 1 µg of the EpCAM BiTE could protect all six mice from challenge with SW480 colon carcinoma cells and even eliminate growth in some mice with established tumors. This same study discovered that the bsAb could utilize tumor infiltrating T-cells to eliminate tumors engrafted in mice when isolated directly from patients at only 5 µg/mouse [103]. This experiment, where three of six mice treated with the BiTE survived challenge, proved that this molecule could utilize a patients' own T-cells to modulate an antitumor effect.

Early generation CD19 BiTEs showed similar promising results. First created in 2000, Loffler et al. demonstrated that the CD19 BiTE could retarget T-cells to lymphoma cells, eliciting significant lysis at low E:T ratios (2:1 or 4:1) at concentrations between 10 and 100 µg/ml, a 10–100-fold lower concentration than even the EpCAM BiTE and 1–10,000-fold lower than a quadroma CD19×CD3 bsAb [71]. Interestingly, short-term addition of IL-2 to PBMCs did not drastically enhance the lysis of B-cells by the CD19 BiTEs, although long-term exposure of IL-2 resulting in LAK effector cells enhanced the EC₅₀ by tenfold [31, 72]. Even at E:T ratios of 1:4, the CD19 BiTE could eliminate all CD19+ B-cells at a low concentration of 5 ng/ml [72]. An in vivo study found that, similar to the EpCAM BiTE, the CD19 BiTE could protect mice from tumor challenge and even eliminate large established tumors [32]. The BiTEs appear to require T-cells to release perforin to mediate lysis, as cells treated with the perforin inhibitor Oncomycin A (OMA), had significantly reduced lysis [38].

3.4.2 MT103 (Blinatumomab)

Learning from previous designs, Micromet designed and created MT103, the CD19×CD3 BiTE currently in clinical trials. MT103 exhibited significant preclinical potency to CD19+ autologous B-cells, with an EC₅₀ of 130 µg/ml with human PBMCs and 150 µg/ml with chimpanzee PBMCs [104]. When the BiTE was given to chimpanzees, the molecule was well tolerated, with an increase in Th1 cytokines and T-cell activation. After a dose of 0.1 µg/kg in chimpanzees, MT103 peaked around 1 ng/ml and reduced to 50 µg/ml within 10–24 h after treatment [104]. As MT103 can induce the release of the Th1 cytokines IFN-γ, TNF-α, and IL-2, it would most likely require coadministration with an anti-inflammatory agent. Therefore, Micromet analyzed the functionality of MT103 with the clinically used

anti-inflammatory agent dexamethasone [17, 19]. Fortunately, the anti-inflammatory agent did not reduce lysis or T-cell activation but did attenuate cytokine release [17]. When compared to a CD19×CD3 tandem, MT103 was 1,000 times more potent, when unstimulated T-cells were used, highlighting for the first time, that the BiTE format was more potent than similarly designed recombinant bsAbs [84]. Another comparison study found that MT103 mediated 150–450 times more efficient lysis than rituximab to CD19+ CD20+ cells but found that even in optimal conditions for rituximab, MT103's addition could still enhance tumor cell apoptosis [25]. Even with extremely low E:T ratios, MT103 was capable of inducing significant cell death, implying that T-cells can mediate serial killing of target cells [25].

These encouraging results led to a phase I trial for MT103 for patients with non-Hodgkin's B-cell lymphoma. Of the 38 patients treated, 11 exhibited objective responses, with four CRs and seven PRs. All six patients treated at the highest dose of 0.06 mg/m² showed a response to treatment. Overall, the adverse events were mild, including chills, pyrexia, lymphopenia, and leukopenia and occurred in the first week of treatment. No antidrug antibodies were seen in any of the 38 patients [10]. A phase II trial, published in 2011, demonstrated an 80 % response rate in 21 patients with adult B-cell lineage ALL, treated with a dose of 0.015 mg/m². After a single cycle of MT103, 16 of 20 evaluable patients were switched from minimum residual disease (MRD) positive status to negative. As MRD is a common indicator of relapse and these patients were heavily pretreated, these results are promising. Overall, the estimate of relapse-free survival was 78 % [114]. As in the phase I trial, MT103 was well tolerated, with similar adverse events. A secondary analysis of the immunological responses to MT103 revealed that most patients responded with similar trends after treatment. Within a single day, the number of circulating B-cells dropped to one B-cell/μL of blood, and did not increase throughout the treatment cycles. The percentage of activated CD8+ and CD4+ T-cells increased from 19.47 to 48.78 % and 12.32 to 35.63 %, respectively, elucidating MT103's potent effects on T-cells. Fortunately, no patient developed HAMA [64]. With these results, MT103 is currently being evaluated in six phase I or phase II clinical trials, and phase III trials results are awaited with interest.

3.4.3 MT110

Similar to MT103, Micromet used previous EpCAM BiTE designs to enhance the potency of the bispecific molecule being tested in clinical trials. The resulting BiTE, MT110, is ~55 kDa, can redirect PBMCs to tumor cells with an EC₅₀ of 230 μg/ml, can eliminate tumor initiating cells and is not affected by circulating levels of EpCAMs ectodomain, EpEx [18, 19, 48, 91]. MT110 was also capable of killing primary pancreatic cells at concentrations of 1–100 ng/ml [23]. In vivo treatment of 1 μg MT110 resulted in the complete prevention of tumor growth in both challenge and established tumors [18]. Interestingly, MT110 was able to induce tumor elimination in mice utilizing only the T-cells that infiltrated the tumor before removal from patients, again elucidating the potency of retargeting T-cells to fight cancer.

In an attempt to determine if an EpCAM BiTE would be toxic to normal EpCAM expressing cells, Brischwein and colleagues performed a series of preclinical studies using a murine EpCAM BiTE analog in immune-competent mice. MuS110 could recognize murine CD3 and murine EpCAM and has an EC_{50} similar to MT110, albeit slightly worse [2, 19]. At doses of 15 $\mu\text{g}/\text{kg}$, MuS110 was well tolerated in mice and was capable of inhibiting syngeneic tumor growth. Increasing the dose to 50 $\mu\text{g}/\text{kg}$ or 500 $\mu\text{g}/\text{kg}$ resulted in cytokine release syndrome or death in mice, respectively [2, 3]. In the 15 $\mu\text{g}/\text{kg}$ treated mice, MuS110 caused a fivefold drop in B-cell numbers and an eightfold drop in T-cell numbers [3]. Unlike humans, mice have EpCAM+lymphocytes, which might be contributing to side effects such as lymphopenia. Therefore, it is possible that initially eliminating this subpopulation of cells could enhance the tolerability of MuS110. Initial low doses of 0.4–10 $\mu\text{g}/\text{kg}$ tolerized mice to doses of MuS110 up to 500 $\mu\text{g}/\text{kg}$ [3, 4]. MuS110 did not damage EpCAM+tissues or organs and long-term dosing did not result in T-cell anergy, strengthening the clinical utility of an EpCAM BiTE. All of these results led to an ongoing phase I clinical trial in patients with EpCAM+solid tumors.

3.4.4 Other BiTEs

The remaining BiTEs in clinical trials target CEA, EphA2, EGFR, CD33, and MCSPs. The CEA BiTE was found from a panel of CEA BiTEs created from three CEA mAbs and thirteen scFvs directed against CEA, all with the same CD3 binding domain. The most potent protein, MT111, had an EC_{50} of 200–500 pg/ml with unstimulated PBMCs and an EC_{50} of 2.3 pg/ml using stimulated human T-cells. It inhibited tumor xenograft growth and had similar lysis across 12 different CEA expressing tumor lines [73]. As many patients with CEA+tumors have circulating soluble CEA (sCEA), it was important to discover that sCEA did not inhibit tumor cell lysis by MT111 [73, 88]. On top of this, a murine analog of MT111 was capable of inhibiting metastatic lung lesions in a syngeneic mouse model [73]. Both of these findings underscore the clinical potential of this BiTE.

EphA2 is a tyrosine kinase receptor that is often overexpressed in aggressive epithelial cancers. The EphA2 BiTE developed by Micromet is unique from others in its class, as it only recognizes EphA2 on malignant cells, sparing any T-cell elimination of normal cells. The BiTE was capable of eliminating tumor cells at EC_{50} concentrations of about 6 ng/ml and could mediate maximal lysis at an E:T ratio of 1:5 with 7 ng/ml of the bsAb. Confirmatory results were seen in vivo [42]. The remaining BiTEs targeting EGFR, CD33, and MCSPs offer promising further preclinical and clinical results. C-BiTE and P-BiTE, developed from the binding domains of cetuximab and panitumumab, two FDA approved EGFR mAbs, were able to lyse KRAS- and BRAF-mutated colorectal cells [74]. The MCSP BiTE recognizes the D3 domain of the heavily glycosylated human melanoma chondroitin sulfate proteoglycan (MCSP) [13]. The bsAb-mediated significant lysis of 33

MCSP+melanoma cell lines with PBMCs isolated from healthy and melanoma patients, albeit reduced with the latter cell sources [115]. The CD33 BiTE, MT114, exhibits similar potent lysis, with EC_{50} values as low as 5 pg/ml and mediates lysis across numerous CD33+ AML cell lines [29, 63].

4 Imaging

There is an evolving area of the tumor immunology field that is utilizing bsAbs for cancer imaging. Since the second target can be engineered to bind to a radiolabeled agent, these bsAbs are simpler to create than radiolabeled mAbs. One of the most common methods for creating radiolabeled bsAbs is the dock-and-lock (DNL) technique. Originally developed in 2006, the DNL method can be used to efficiently create bsAbs for any target but as of 2012 was entirely devoted to the creation of radiolabeled bsAbs [97]. TF2, which targets CEA and the hapten histamine-succinyl-glycine (HSG), is currently undergoing three phase I trials and one phase I/II trial for imaging colorectal or lung cancers [78]. After injection and clearance of the bsAb, ^{99m}Tc -labeled hapten is infused into the patient, resulting in radioactive labeling of only bsAb bound cells. TF4 and TF10 are two other DNL bsAbs directed against CD20×HSG hapten and MUC1×HSG hapten, respectively [35, 108].

5 Conclusions

The clinical efficacy of catumaxomab and blinatumomab highlight the potential of bispecific antibody therapy modalities. The progress seen 25 years following the production of the first such molecules augurs well for the future of the field (Table 1). Learning from previous designs and trials, researchers have developed molecules that overcome problems like the development of HAMA, by creating bsAbs that work at increasingly low concentrations, precluding the need for excessive doses or humanization. First generation bsAbs required such high ratios of effector to tumor cells that could never translate into clinical efficacy. The efficiency of newer compounds permits them to be used at low concentrations that require potentially physiological effector: target ratios. Moreover, by targeting hematologic malignancies, the potential barriers of access of effector cells to tumor targets may be minimized. Challenges remain; the need for prolonged continuous infusion makes BiTEs inconvenient and expensive for patients. Additional modifications to promote prolonged half-lives may be required for these reagents to achieve their full potential. Moreover, the ability of BiTEs to effectively treat solid human tumors in the clinical setting remains unproven. However, the presence of these challenges should not obscure the fact that bispecific antibodies can work to effectively treat cancer. The challenge to the field is to build upon the exciting findings for the benefit of patients with diverse forms of cancer.

Table 1 A list of bsAbs that have entered clinical trials

Name	Design	Target	Species	Indication	Phase	References
MDX-210	(Fab) ₂	HER2 × CD64	Murine	Breast	I	[116]
MDX-H210	(Fab) ₂	HER2 × CD64	Humanized	Breast	I	[94]
MDX-447	(Fab) ₂	EGFR × CD64	Humanized	EGFR tumors	I	[34]
2B1	Quadroma	HER2 × CD16	Murine	HER2 tumors	I	[15]
HRS-3/A9	Quadroma	CD30 × CD16	Murine	Lymphoma	I/II	[44]
SHR-1	Quadroma	CD19 × CD3	Rat/murine	Lymphoma	I	[26, 27]
HEA125 × OKT3	Quadroma	EpCAM × CD3	Murine	Malignant ascites	I	[77]
Catumaxomab	Triomab	EpCAM × CD3	Rat/murine	Malignant ascites	EU approval/III	[47]
Ertumaxomab	Triomab	HER2 × CD3	Rat/murine	HER2 tumors	I/II	[59]
Bi20	Triomab	CD20 × CD3	Rat/murine	Lymphoma	I	[20]
MEHD794A	Two-in-one	HER3 × EGFR	Human	Solid tumors	I/II	NCT01577173
CVX-241	CovX body	VEGF-A × Ang-2	Human	Solid tumors	I	NCT01004822
rM28	TaFv	NG2 × CD28	Murine	Melanoma	I/II	NCT00204594
MM-111	TaFv-albumin	HER2 × HER3	Human	HER2 tumors	I	NCT00911898
MT103	BITE	CD19 × CD3	Murine	Lymphoma	II	[114]
MT110	BITE	EpCAM × CD3	Murine	EpCAM tumors	I	NCT00635596

Citations represent the most recently published trial. If a reference isn't cited, the clinical trial is currently in progress

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