# Multifunctional receptor-targeting antibodies for cancer therapy

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**During the past decade, monospecific antibodies targeting cell-surface receptors in different tumour types have achieved substantial success and have been at the forefront of cancer treatment. However, redundant signalling and crosstalk between different pathways within tumour cells and between tumour cells and their microenvironment can limit the efficacy of receptor-targeted monospecific-based therapies. Advances in antibody engineering technologies have enabled strategies that simultaneously target multiple receptors to circumvent the limitations of conventional monospecific therapies and achieve enhanced therapeutic efficacy. In the past 5 years, a range of multifunctional, receptor-targeting, antibody-based molecules have emerged, which allow targeting of multiple surface receptors on tumour cells and endothelial or immune cells in the tumour microenvironment. In this Review, we discuss the rationales and strategies for the use of multifunctional receptor-targeting antibodies, their mechanisms of action, and the promises and challenges they hold as cancer therapeutics. This knowledge provides opportunities to improve current targeted therapy outcomes for patients with cancer.**

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## Introduction

Antibody-based therapeutics have been one of the most exciting and promising specialties of cancer therapy in the past two decades. Despite some notable successes with therapeutic antibodies such as trastuzumab targeting HER2 in metastatic breast cancer,[1] rituximab targeting CD20 in B-cell lymphoma,[2] and ipilimumab targeting CTLA-4 in patients with melanoma,[3] therapies targeting single antigens with monospecific antibodies have shown limited efficacy in patients with cancer. Intrinsic and acquired resistance often occurs because alternative receptors or pathways are activated in targeted cancer therapy.[4] In addition to these mechanisms, the tumour microenvironment also plays a part in tumour progression and resistance to therapies, and has become an attractive therapeutic target.[5] With advances in the understanding of the complex biology of tumours, multifunctional receptor-targeting antibodies—which aim to simultaneously target different pathways within tumour cells and within their microenvironment to exert immune modulation function—are regarded as an exciting avenue for next-generation cancer therapy.

The basic structure of conventional antibodies is well conserved in mammals: they are polypeptide tetramers consisting of two identical pairs of heavy and light chains linked by interchain disulphide bonds (figure 1A). By contrast with conventional antibodies, heavy chain-only antibodies[9] are about 10–12 kDa smaller because they do not have a CH1 domain (figure 1B) and can recognise the antigen with a single domain by contrast with the $V_H$–$V_L$ pair in conventional antibodies, antigen-binding fragments, or single-chain variable fragments (consisting of two variable domains, one each from heavy and light chains; figure 1C). The recombinant expression of the $V_{HH}$ yields small and soluble single domain antibody fragments, which have dimensions in the nanometre range and have been defined as nanobodies (figure 1D). Compared with conventional antibodies, the different antibody fragments are smaller in size and can penetrate to tumour tissues inaccessible to full-size antibodies. However, the small size of antibody fragments limits their efficacy in therapeutic applications because of their short serum half-life due to rapid renal clearance.[10] Antibody fragments that are devoid of the Fc domain require conjugation to a functional moiety to induce antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity.[11]

Preclinical and clinical studies in the past decade have revealed that monospecific antibodies for cancer have limited targets and efficacy.[12] The development of bispecific and multispecific antibody approaches have become a key technology, empowering a new generation of antibody-based therapeutics in the past few years.[12] This is a unique approach and is distinct from the traditional monoclonal antibody or antibody combination approaches because it allows multiple targeting, which reduces toxicity and can bring immune effector cells into close proximity with cancer cells, ultimately enhancing therapeutic efficacy and preventing drug resistance.

The concept of recombinant bispecific antibodies was first described more than two decades ago by fusing an anti-dansyl single-chain antibody to the hinge or CH3 domain of anti-dextran IgG.[13] A range of bispecific or multispecific antibodies were then generated on the basis of this initial concept, in which the N-termini or C-termini of antibodies were fused with additional binding entities such as single-chain or disulphide-stabilised fragments of variable regions or antigen-binding fragments.[14] By contrast with these bispecific formats, a strategy based on the modifications of the light-chain sequence generated the first so-called two-in-one antibody, which binds HER2 and VEGF at a single-antigen binding site.[15] This design, therefore, has all the favourable IgG attributes for therapeutic development. More than 35 different formats of bispecific and multispecific antibodies and their derived constructs are under early development by several pharmaceutical companies.[12] By contrast with bispecific antibodies and

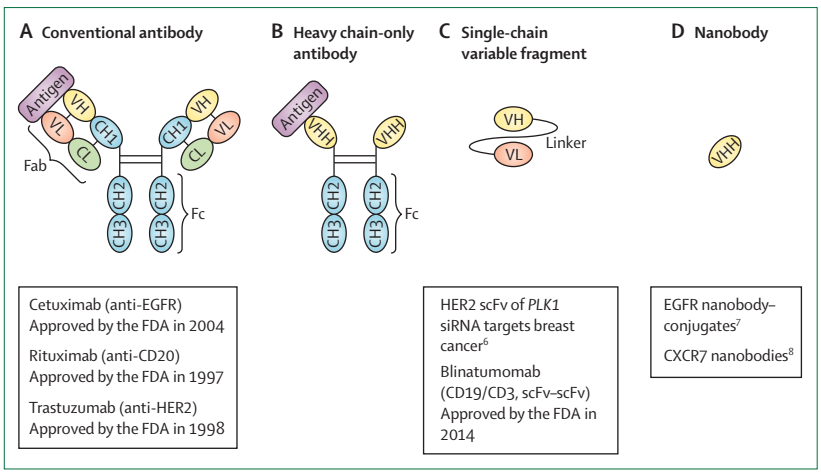


***Figure 1:*** **Domains of different types of antibodies**
FDA=US Food and Drug Administration. scFv=single-chain variable fragment.

multispecific antibodies, different antibodies have been conjugated with cytotoxic drugs, toxins, or radionuclides, and kill tumour cells directly. Both bispecific and multispecific antibodies and antibody conjugates have been designed to target immune cells and tumour angiogenesis in the tumour microenvironment. In this Review, we will discuss present challenges in targeted cancer therapy in the clinic and recent advances of those approaches targeting the tumour and its microenvironment in both preclinical cancer models and clinical settings. The focus will be on the rationale for each of these approaches, their mechanisms of action, and the promises and challenges of these drugs as cancer therapeutics.

## Bispecific and multispecific antibodies targeting multiple receptor tyrosine kinases in tumour cells

Crosstalk between different receptor tyrosine kinases (RTKs), such as HER family members, MET, VEGFR, and IGF-1R, are known to cooperatively promote cancer progression and drug resistance (figure 2A).[16] The HER2 monoclonal antibody trastuzumab has led to improved outcomes in patients with HER2-positive breast cancer. However, most patients with metastatic HER2-positive breast cancer develop resistance within a year of treatment.[17] Activation of multiple RTK pathways has been shown to contribute to the development of trastuzumab resistance.[18] Similarly, a substantial proportion of patients with lung or colon cancer do not respond to anti-EGFR drugs and acquire resistance after initial benefit.[19] For example, the gain of MET amplification in patients with lung cancer is one of the mechanisms leading to acquired resistance to the small-molecule EGFR inhibitors gefitinib and erlotinib.[20] This complexity of tumour signalling implies the necessity of targeting multiple RTKs for effective cancer therapeutics. The variety and versatility of the available multi-targeting formats provide many opportunities for the enhancement of antibody-based cancer therapy via engagement of multiple cell-surface receptors.[12] In addition to the fact that the efficacy of different monotargeting therapies is often improved by their combination, studies have reported that in some cancer contexts, use of a single drug for simultaneous targeting of cell-surface receptors could be superior in terms of dosage, efficacy, and binding affinity, compared with monospecific antibodies.[21,22] At present, at least 14 multi-targeted tyrosine kinase inhibitors targeting RTKs are available in the clinic for patients with cancer,[23] whereas most of the bispecific and multispecific antibodies are still in the early stages of development.

### Co-targeting HER family members (EGFR/HER2, EGFR/HER3, HER2/HER3)

The HER family (figure 2B) is one of the most robustly investigated families of RTKs for multispecific antibody targeting. These structurally related receptors are of interest because of their overexpression or overactivation in various solid cancer types and their capacity for crosstalk, which might have a key role in their acquired resistance to monospecific antagonistic antibody therapy.[16] Therefore, simultaneous targeting of multiple HER family members (figure 2C) has the potential to substantially improve outcomes over monospecific therapy, especially when considered in specific cancer contexts.

HER2 is regarded as the preferred heterodimerisation partner for all other HER family receptors.[24] Co-expression of HER2 and EGFR has been reported in several cancers and has a prognostic value in non-small-cell lung cancer (NSCLC)[25] and breast cancer.[26] The heterodimerisation of HER2 and EGFR is known to induce a potent tyrosine kinase activation signal compared with EGFR homodimerisation,[25] suggesting that targeting both HER2 and EGFR would be an advantageous treatment approach. At present, the US Food and Drug Association (FDA)-approved dual tyrosine kinase inhibitor, lapatinib, which targets EGFR and HER2, is being used in combination therapy for HER2-positive breast cancer. The anti-EGFR/HER2 bispecific antibody (TC-BsAb)—engineered by the fusion of $V_H$ and $V_L$ of trastuzumab (anti-HER2) to the N-terminus of the $V_H$ and $V_L$ of cetuximab (anti-EGFR)—retains the binding affinity of both parental monoclonal antibodies and has the ability to downregulate both EGFR and HER2.[21] This anti-EGFR/HER2 bispecific antibody has been shown to have enhanced therapeutic efficacy compared with either trastuzumab or cetuximab or their combination in a preclinical model of breast cancer.[21] These findings imply that bispecific antibodies targeting EGFR and HER2 should be further developed and assessed in other types of cancer in which EGFR and HER2 overexpression and their signalling crosstalk are dominant in tumour progression.

HER3, though lacking an active kinase domain, is capable of crosstalk with EGFR and other family members

via heterodimerisation and co-activation of heterodimerised receptors, and HER3 inactivation has been associated with sensitivity to EGFR antagonists in patients with NSCLC[27] and patients with colorectal and pancreatic cancers.[28] Accordingly, a bispecific antibody targeting EGFR and HER3 is under development and clinical assessment at present. By contrast with most bispecific drugs, which are constructed by linking two monospecific antigen-binding modules, a two-in-one antibody against EGFR and HER3, MEHD7945A, was generated through two steps: generation of high-affinity anti-EGFR antibodies via a phage-displayed antigen-binding fragment library with diversity restricted to heavy chain complementarity-determining regions, and then the addition of HER3 binding to the anti-EGFR antibody via mutations in the light-chain complementarity-determining regions.[22] MEHD7945A has two identical antigen-binding fragment arms and is capable of engaging two EGFRs, two HER3, or one EGFR and one HER3 simultaneously on the cell surface. The unique two-in-one feature of MEHD7945A was reported to have a superior anti-proliferative effect and broader therapeutic efficacy in vivo in 12 murine xenograft models compared with single antibodies, especially in those tumours where both EGFR and HER3 contribute to tumour growth.[22] In the same study,[22] MEHD7945A has also been shown to be highly effective in inducing antibody-dependent cell-mediated cytotoxicity. Several clinical trials of MEHD7945A are in progress, including phase 2 trials in recurrent head and neck cancer (NCT01577173), MEHD7945A plus FOLFIRI (leucovorin, fluorouracil, and irinotecan) as a second-line treatment in *KRAS* wild-type metastatic colorectal cancer (NCT01652482), and a phase 1b study of MEHD7945A in combination with the MEK inhibitor cobimetinib in patients with locally advanced solid *KRAS*-mutant tumours (NCT01986166).

HER2 does not have a high-affinity ligand, but in the context of HER2 overexpression, forms a heterodimer with HER3, which contributes greatly to the maintenance of oncogenic signalling via activation of the PI3K pathway.[29] The HER2/HER3 heterodimer contains a binding site that has an affinity 100 times higher for the ligand heregulin than the affinity of HER3 alone,[30] and forms a ligand–dimer complex that is difficult to impede via monospecific antibodies. With an improved understanding of HER3 signalling in the context of HER2-amplified tumours such as breast cancer,[31] bispecific antibodies against HER2 and HER3 have been engineered. MM-111, a bispecific antibody that consists of a fusion between single-chain variable fragment binding arms of anti-HER2 and anti-HER3,[32] has been shown to effectively form a trimeric complex with the HER2/HER3 dimer, resulting in superior inhibition of the PI3K pathway compared with lapatinib (EGFR and HER2) and pertuzumab (HER2).[32] These exciting data in preclinical studies have led to phase 1 and 2 clinical trials in HER2-positive breast cancer and gastro-oesophageal cancer with MM-111 alone or in combination with multiple treatments such as trastuzumab, cisplatin, capecitabine, lapatinib, paclitaxel, and docetaxel (NCT01097460, NCT01304784, NCT00911898, NCT01774851; table).

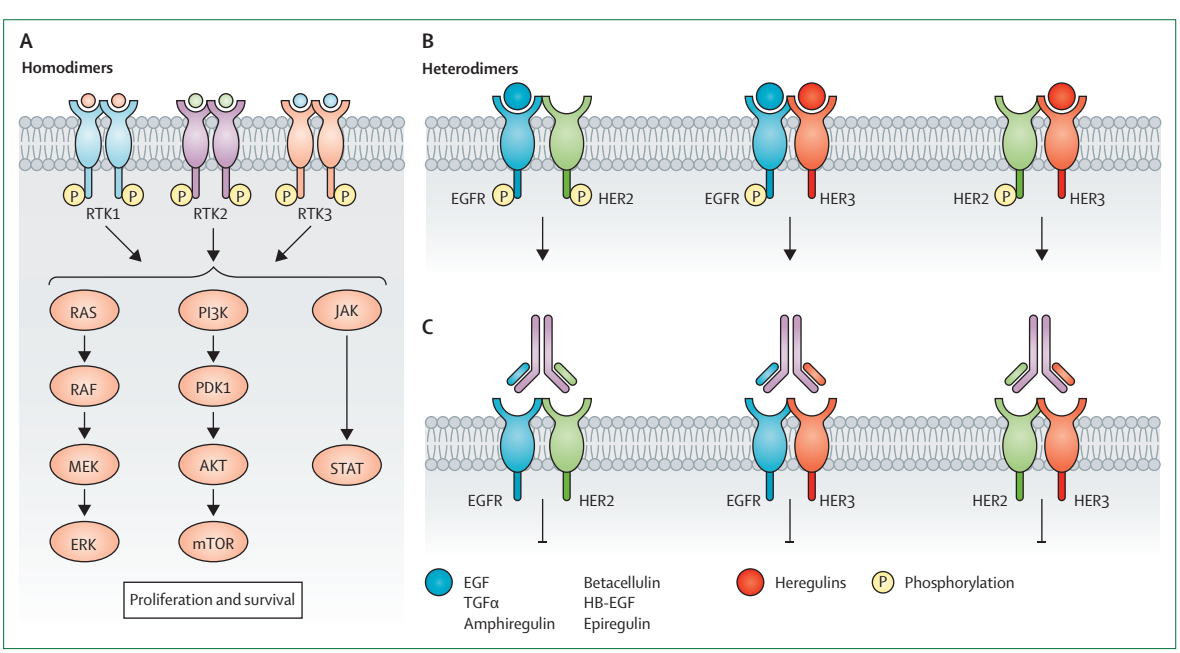


***Figure 2:*** **Co-targeting HER family members by bispecific antibodies**
(A) Common downstream signalling pathways shared by different receptor tyrosine kinases contribute to tumour cell proliferation and survival. (B) Heterodimerisation of HER family members contributes to the maintenance of oncogenic signalling. (C) Blocking oncogenic signalling by use of bispecific antibodies to target different members of HER family.

### Co-targeting HER family members and other RTKs

The crosstalk and oncogenic shift between HER family members and other RTKs has been shown to be implicated in the development of tumour-cell resistance to single-agent anti-EGFR or anti-HER therapy.[16] Therefore, several strategies have been developed to synthesise bispecific and multispecific antibodies to target both HER family members and other oncogenic RTKs.

Several studies have reported that multiple compensatory mechanisms are enacted by IGF-1R in response to antagonistic EGFR treatment.[33–35] Combined blockade of EGFR and IGF-1R has shown improved therapeutic efficacy over the targeting of either receptor alone in preclinical tumour models.[36] These studies provide a rationale for the development of bispecific antibodies that simultaneously target both EGFR and IGF-1R. By attaching an anti-IGF-1R single-chain variable fragment to a full-length IgG capable of binding to both EGFR and the mutated EGFRvIII, the tetravalent bispecific antibody EI-04 has been shown to target EGFR and IGF-1R simultaneously, blocking both AKT and ERK signalling, and resulting in enhanced anti-tumour efficacy compared with monospecific antibodies and their combination in BxPC3 tumour cells (pancreatic cancer) and GEO (colon cancer) tumour xenograft models.[37] However, EI-04 did not maintain the native IgG capacity for mediating antibody-dependent cell-mediated cytotoxicity, and showed a diminished effect on receptor down-regulation compared with the parental monoclonal antibody C06.[37] By using a different approach other than EI-04, several variants of bispecific anti-EGFR/IGF-1R antibodies have been created by the fusion of IGF-1R monoclonal antibodies with single-chain variable fragments of EGFR monoclonal antibodies at the N-terminal or C-terminal of light or heavy chains. These variants show improvements in terms of yield and stability, and affect both antibody-dependent cell-mediated cytotoxicity and receptor internalisation.[38] Despite promising preclinical data and a strong rationale, the strategy for combining EGFR and IGF-1R inhibitors has not been translated into effective treatment in most clinical trials. The only promising data are from a phase 2 trial,[39] which used a combination of the IGF-1R monoclonal antibody R1507 and erlotinib and showed benefit to patients with *KRAS*-mutated advanced NSCLC. All of the clinical trials targeting EGFR and IGF-1R with negative results did not have enough patient selection and predictive biomarkers. Collection of biomarker data for dual targeting of EGFR and IGF-1R by bispecific antibodies is important to gain meaningful information from future trials.

Activation or amplification of MET is a major escape route (oncogene shift) for EGFR-targeted therapies. Previous studies have shown that MET is involved in both de-novo and acquired resistance of NSCLC to

| | Target | Source | Phase in clinical trials (year) | National clinical trial number | Targeted cancer types |
|---|---|---|---|---|---|
| Solitomab | CD3/EpCAM | Mouse scFv–scFv fusion | 1 (2007) | NCT00635596 | Advanced solid tumours |
| Ertumaxomab | CD3/HER2 | Mouse and rat hybrid | 2 (2006) | NCT00351858 | Breast cancer |
| MDX-447 | EGFR/CD64* | Humanised | 1 (2000) | NCT00005813 | Recurrent or refractory glioblastoma |
| TF2 | CEA/HSG | Humanised | 1/2 (2010) | NCT01221675 | CEA-expressing NSCLC |
| rM28 | CD28/MAPG | Dimer of scFv–scFv fusion | 1/2 (2005) | NCT00204594 | Metastatic melanoma |
| CD20Bi | CD3/CD20 | Hybrid, muromonab-CD3 and chimeric rituxan (CD20) | 1 (2009) | NCT00938626 | Multiple myeloma |
| F6-734 | CEA/DTPA | Mouse anti-CEA Fab2 (F6) and Fab2 of 734 (anti-DTPA In IgG1) | 2 (2007) | NCT00467506 | Medullary thyroid carcinoma |
| MGD006 | CD123/CD3 | Humanised | 1 (2014) | NCT02152956 | Relapsed or refractory acute myeloid leukaemia |
| MEHD7945A | EGFR/HER3 | Human IgG1 | 2 (2012) | NCT01577173 | Head and neck cancer |
| MM-111 | HER2/HER3 | Human scFv–scFv-HSA | 2 (2013) | NCT01774851 | HER2-positive oesophageal carcinomas |
| FBTA05 | CD3/CD20 | Rat and murine hybrid | 1/2 (2010) | NCT01138579 | B-cell lymphoma |
| BAY2020112 | CD3/PSMA | scFv–scFv | 1 (2012) | NCT01723475 | Prostate cancer |
| LY3164530 | EGFR/MET | Not known | 1 (2014) | NCT02221882 | Advanced or metastatic cancer |
| RO6958688 | CEA/CD3 | Not known | 1 (2014) | NCT01723475 | Locally advanced or metastatic cancer |
| DT2219ARL | CD22/CD19 | Diphtheria toxin scFv–scFv | 1 (2015) | NCT02370160 | Relapsed or refractory B-lineage leukaemia |
| MGD007 | gpA33/CD3 | Humanised | 1 (2014) | NCT02248805 | Relpased or refractory metastatic colorectal carcinoma |
| MOR209/ES414 | PSMA/CD3 | Humanised | 1 (2014) | NCT02262910 | Metastatic castration-resistant prostate cancer |
| OMP-305B83 | DLL4/VEGF | Humanised | 1 (2014) | NCT02298387 | Previously treated solid tumours |

scFv=single-chain variable fragment. HSG=histamine succinyl glycine. NSCLC=non-small-cell lung cancer. DTPA=diethylenetriamine pentaacetic acid. HSA=human serum albumin. PSMA=prostate-specific membrane antigen. gpA33=glycoprotein A33. DLL4=delta-like ligand 4. *Also known as FcgR I.

***Table:*** **Bispecific antibodies in clinical trials**

tyrosine kinase inhibitors,[20] and that EGFR inhibition induces HGF-stimulated tumour cell invasion.[40] In view of the fact that bivalent antibodies targeting MET induce receptor dimerisation and mimic HGF agonism,[41] a one-armed MET antibody (onartuzumab) has been developed[42] and has been shown to inhibit HGF/MET axis-addicted tumour growth in different preclinical models.[43,44] On the basis of these studies, a phase 2 study was designed in which patients with NSCLC who are MET-positive were given onartuzumab and erlotinib and showed improved progression-free survival.[45] However, the phase 3 trial (NCT01456325) in patients with metastatic NSCLC who had high MET expression levels in tumour tissue did not show survival benefits after onartuzumab and erlotinib treatment. This outcome could be attributed to the use of MET expression levels in the tumour tissue as a biomarker to stratify patients and suggests a need for the consideration of the use of both HGF and MET expression levels and other parameters for patient selection.

On the basis of similar rationale of co-targeting EGFR and MET, the bispecific antibody MetHer1 was created via cloning fragments of variable regions of cetuximab into an IgG1 antibody backbone bearing an anti-MET single-chain antigen-binding fragment fused at the C-terminus of one of the heavy chains. MetHer1 is a glycosylated human IgG1 antibody that maintains immune effector functions. MetHer1 is superior to both parental monoclonal antibodies and their combination at low doses and has a potent anti-tumour effect in vivo.[40] Although the bispecific antibody has shown greater efficacy than the monospecific antibody, the acquired resistance by different compensatory mechanisms involving multiple RTKs would require multispecific drugs that simultaneously target multiple signalling pathways.

The tetra-specific four-in-one antibody CRTB6 is the first antibody capable of binding EGFR, HER2, HER3, and VEGF simultaneously. CRTB6 has been shown to be more potent than bispecific antibodies in both in-vitro and in-vivo preclinical models.[46] This could be due to its ability to target four RTKs simultaneously. Since CRTB6 is an IgG-like antibody, the antibody-dependent cell-mediated cytotoxicity effect should also be assessed for anti-tumour efficacy.

## Antibody–nanobody conjugates targeting tumour cells

Despite several advantages of antibodies compared with other treatments for cancers, only 15 unmodified therapeutic antibodies have been approved by the FDA as cancer drugs, underscoring the difficulty of developing clinically effective antibodies. However, provided that tumour specificity is ensured in biodistribution studies, cancer antibodies can be used as delivery vehicles of toxic payloads. To enhance cytotoxicity, therapeutic antibodies have been conjugated to toxins, cytotoxic drugs, and radionuclides.[47,48] Covalent coupling of a small-drug molecule to an antibody is an important step to create antibody–drug conjugates (ADC), which would deliver toxins to tumour tissues, enhance the solubility of hydrophobic drug compounds, and increase serum half-life by prevention of renal clearance.[47] Most conjugated drugs target one of three cellular structures: tubulin, DNA, or RNA. Among early ADCs, FDA-approved chemotherapeutics such as doxorubicin,[49–51] vinca alkaloids,[52–54] and methotrexate[55,56] did not show significant anticancer activity in clinical studies. By contrast, the tubulin toxins maytansinoids[57,58] and auristatins[59] and the DNA toxin calicheamicin[60] have shown promising results as drug conjugates of ADC in several clinical studies published within the past 5 years, producing higher potencies than conventional chemotherapy alone. At present, more than 30 ADCs are in clinical trials and four ADCs are being marketed. Ibritumomab tiuxetan[61] (conjugated to $^{90}$Y) and iodine-131 tositumomab[62] (conjugated to $^{131}$I) are made of two murine anti-CD20 antibodies and are being used to treat relapsed or refractory follicular lymphoma. Two ADCs have recently been marketed: brentuximab vedotin, an anti-CD30 antibody conjugated to microtubule toxin auristatin, which was FDA-approved in 2011,[63] and ado-trastuzumab emtansine, an anti-HER2 antibody conjugated to microtubule toxin maytansine, which was FDA-approved in 2013.[64] Brentuximab vedotin is used to treat Hodgkin's lymphoma and anaplastic large-cell lymphomas, and ado-trastuzumab emtansine is used to treat metastatic HER2-positive breast cancer by inducing cancer cells to mitotic catastrophe. In addition to efforts to develop an ADC against epithelial antigens, the strategy of future ADC development should also be directed to target cancer-initiating cells and vascular and stromal components in the tumour microenvironment.

Compared with conventional antibodies, nanobodies have high stability and solubility, are suitable for production of multivalent and multi-specific formats with flexible linker design, and are well expressed in microorganisms.[11] Nanobodies specific to cancer-related antigens such as EGFR,[65] HGF,[66] and the chemokine receptor CXCR7[8] have been isolated, and nanobody-based anti-cancer therapies are emerging.[67] However, the small size of nanobodies restricts their efficacy in therapeutic applications because of their short serum half-life due to rapid renal clearance. Thus, modified nanobodies have been developed by fusing nanobodies to albumin, immunoglobulin, or the Fc region and by pegylation to extend serum retention time by increasing molecular weight or increasing the interaction of the nanobody with the Fc receptors of monocytes.[68–70] Similar to traditional antibody conjugates, nanobody conjugates such as EGFR nanobody-TRAIL[7] and EGFR nanobody-pseudomonas exotoxin[71] have been tested in preclinical tumour models and have shown better

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efficacy than using the nanobody by itself. In a previous study, we characterised an EGFR-specific nanobody and showed that EGFR-specific nanobodies released from therapeutic stem cells specifically localise to tumours and inhibit EGFR signalling, resulting in reduced glioblastoma growth and invasiveness in vitro and in vivo.[7] Similar to EGFR-specific nanobodies, EGFR nanobody-TRAIL specifically binds to EGFR and competes with the EGF ligand binding to EGFR, which results in a reduced activation of EGFR and its downstream signalling pathways. By comparison with cetuximab and EGFR-specific nanobodies, therapeutic stem-cell-delivered EGFR nanobody-TRAIL has shown much better therapeutic efficacy in a preclinical glioblastoma model.[7] As a bispecific molecule, EGFR nanobody-TRAIL induces apoptosis via activation of death receptors 4 and 5. This process is not affected by the sensitivity of the cell to EGFR antagonists, allowing EGFR nanobody-TRAIL to target a broad spectrum of tumour cells that are resistant to EGFR-targeted therapy. However, the EGFR nanobody-pseudomonas exotoxin blocks protein synthesis by catalysing the inactivation of elongation factor-2. Both therapeutic molecules have shown efficacy in different types of cancer cells that are resistant to EGFR-targeted therapy.

## Multifunctional antibodies co-targeting the tumour and its microenvironment

Many antibody-based therapeutic strategies have been developed to target stromal cells in the tumour microenvironment to either block angiogenesis or enhance anti-tumour immune responses. In particular, blocking immune inhibitory receptors on immune cells allows tumour cells to be subjected to attack from cytotoxic T cells.[72] Antibodies against PD-1 on immune cells have shown high promise in several tumour types in the clinic, including metastatic melanoma, metastatic renal-cell carcinoma, Hodgkin's lymphoma, and NSCLC.[73–75] Two antibodies against PD-1, nivolumab and pembrolizumab, were approved by the FDA in 2014 for the treatment of patients with unresectable or metastatic melanoma. However, a substantial fraction of patients have primary resistance or acquired resistance to anti-PD-1 therapy.[76] The resistance mechanism of tumour cells evading immune detection involves immune-suppressive cytokines and cells in the tumour microenvironment, suggesting that multiple components within tumours and the tumour microenvironment need to be targeted.

### Bispecific antibodies targeting both tumour cells and angiogenesis

During tumour progression, hypoxia and glucose consumption are known to induce angiogenesis within the tumour.[77,78] Both tumour cells and tumour-associated endothelial cells express growth factors and their corresponding receptors, such as EGFR and VEGFR. In a study by Yokoi and colleagues,[79] dual inhibition of EGFR and VEGFR by kinase inhibitors reduced tumour growth and metastasis in a mouse model of colon cancer, suggesting that the EGFR and VEGFR pathways have important roles in regulating tumour progression and neovascularisation. More strikingly, an increase in EGFR expression and loss of ErbB3 expression has been identified in tumour vasculature and provides the rationale to target EGF-induced endothelial cell proliferation in tumour vasculature.[80] Co-inhibition of PDGFRβ and VEGFR has been shown to prevent new blood vessel growth better than VEGFR alone.[81] So far, a few tyrosine kinase inhibitors (axitinib, sorafenib, and sunitinib) targeting both VEGFR and PDGFR are on the market for treating renal cell carcinoma and patients with other types of cancer.[23] Bispecific antibodies—such as single-chain variable fragments dual-targeting PDGFRβ and VEGF-A,[82] and Ang-2-VEGF-A CrossMab, which targets angiopoietin 2 and VEGF-A simultaneously[83]—have been reported to attenuate angiogenesis through inhibition of two distinct pathways and represent an efficient way to target tumour angiogenesis. In addition to EGFR, VEGFR, and PDGFRβ, death receptor-5 has also been characterised as a target in endothelial cells within tumours.[84] Bispecific antibodies against those targets that are shared by tumour cells and tumour-associated endothelial cells have the potential for an enhanced therapeutic efficacy.

### Bispecific and multispecific antibodies targeting both tumour cells and immune cells

Bispecific and multispecific antibodies have been developed that simultaneously target cell-surface receptors on tumour cells and those on immune cells in the tumour microenvironment. Targets include tumour-associated antigens, such as CD19, CD20, carcinoembryonic antigen, and EpCAM;[22,85–88] molecules associated with immune effector cell recruitment and activation, such as CD28 and CD3 on T cells and Fc receptors on immune cells;[85,89,90] and death receptors to induce direct cytotoxicity such as CD95 (also called Fas receptor or APO-1), tumour necrosis factor-related apoptosis-inducing ligand receptor (types 1 and 2—also known death receptors 4 and 5, respectively), and lymphotoxin-beta receptor.[91,92] A range of bispecific antibodies aimed at linking tumour cells and immune cells are undergoing clinical trials at present (table). Among them, blinatumomab—which is bispecific to CD3 and CD19, links CD19-positive blast cells of B-precursor acute lymphoblastic leukaemia to CD3-positive T cells, and promotes perforin-mediated cytotoxicity[93]—has shown promising results in a phase 2 clinical trial in adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia.[94] A phase 3 study of blinatumomab (NCT02013167), targeting the same patients as the phase 2 study, has shown promising

outcomes. In view of the unique mechanism of action and success of blinatumomab in phase 2 and 3 studies, FDA approval of blinatumomab was granted at the end of 2014 under accelerated approval conditions for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukaemia.

### Antibody–cytokine fusion proteins targeting both tumour cells and immune cells

Immunoregulatory cytokines have been well known for their role in anti-tumour immune responses.[95] Under physiological conditions, cytokines exert their functions through autocrine or paracrine pathways, or both, within the tumour microenvironment. Therefore, systemic delivery of cytokines is often accompanied by severe side-effects and insufficient concentration of cytokines at the tumour site.[96] The use of tumour-specific antibody–cytokine fusion proteins allows the accumulation of the tumour-specific fusion proteins within the tumour and the tumour microenvironment, which not only reduces systemic toxicity, but also improves therapeutic efficacy compared with the combined treatment of the antibody and cytokines.[97,98] Several cytokines including interleukin 2, granulocyte-macrophage colony-stimulating factor, interleukin 12, tumour necrosis factor α, and interferon γ have been fused to antibodies to generate antibody–cytokine fusion proteins. For example, interleukin 2 has been fused to diverse antibody formats (IgG, single-chain variable fragment-Fc, single-chain variable fragment, and diabody) against various tumour antigens to target different types of cancers.[99] Preclinical studies testing antibody-fusion proteins of interleukin 2, granulocyte-macrophage colony-stimulating factor, and lymphotoxin α revealed that they induce different populations of anti-tumour immune cells.[100] Previous studies[101,102] have also shown that if the same antibody–cytokine fusion is used in different tumour types, therapeutic effects are exerted from different immune cell populations. These observations suggest that the antibody–cytokine-mediated therapeutic effect via immune cells is tumour and tumour microenvironment dependent. To develop the next generation of antibody–cytokines, good biopharmaceutical properties with efficient tumour targeting and anti-tumour activity should be taken into consideration.

### Tumour-associated macrophages: a novel target

Tumour-associated macrophages are a key part of the tumour microenvironment and during tumour progression acquire M2-like properties and secrete cytokines that promote tumour growth, metastasis, immunosuppression, and invasiveness, and trigger drug resistance in cancer stem and initiating cells.[103,104] Clinical studies of classic Hodgkin's lymphoma and breast cancer have shown that the increased infiltration of tumour-associated macrophages is associated with a poor disease prognosis.[105,106] More interestingly, an in-vitro co-culture study of EGFR-positive tumour cells and monocytes revealed that anti-inflammatory and tumour-promoting mediators are released after loading tumour cells with cetuximab, suggesting that the application of the therapeutic antibody might activate tumour-promoting tumour-associated macrophages in the local tumour microenvironment.[107] These observations might explain the decreased progression-free survival in patients with metastatic colorectal cancer when cetuximab was added to bevacizumab and chemotherapy.[108] Tumour-associated macrophages have also been shown to modulate the efficacy of other anticancer therapies, including chemotherapy, irradiation, and immunotherapy.[109] Together, these studies suggest the importance of targeting tumour-associated macrophages in the tumour microenvironment as part of an integrated strategy of cancer therapy.

Attempts to target tumour-associated macrophages in the tumour microenvironment involve targeting tumour-associated macrophages directly,[110] blocking recruitment chemokines of tumour-associated macrophages,[111,112] or manipulation of the repertoire of cytokines released by tumour-associated macrophages.[113,114] Macrophage colony-stimulating factor (CSF1), and its receptor, CSF1R, regulate macrophages and their progenitors. Inhibition of CSF1R with monoclonal antibody emactuzumab selectively induces apoptosis of CSF1R-positive and CD163-positive M2-like macrophages but not CD80-positive M1-like macrophages in vitro. In one study,[115] blocking CSF1R reduced tumour-associated macrophages in tumour-bearing mice and delayed tumour growth and metastasis. Furthermore, treatment with emactuzumab has been shown to deplete tumour-associated macrophages from the tumour tissue of patients across different cancer types, including endometrial cancer, breast cancer, and colorectal cancer.[115] At present, emactuzumab is in phase 1 clinical trials in solid tumours and pigmented villonodular synovitis (NCT02323191).[116] A different approach such as the use of a tumour-associated macrophage-targeting ligand has also been shown to be effective in a mouse syngeneic model.[117] Together, these studies suggest that targeting M2-like tumour-associated macrophages in the tumour microenvironment is a new approach for tumour-targeted therapy.

## Conclusions

Antibody-based therapeutics for cancer have been at the forefront of cancer treatment during recent decades due to their high specificity and low toxicity. However, FDA-approved monospecific antibodies targeting tumour-selective receptors have shown limited efficacy as single agents in patients with cancer. Improper

patient selection and inappropriate biomarkers are among the major causes behind the negative results reported by trials of these antibodies. Meanwhile, activation of multiple receptor pathways within the tumour cells and crosstalk between tumour cells and the tumour microenvironment also contribute to resistance to single-targeted therapy. In this Review, currently available and in development multifunctional receptor-targeting antibodies have been reviewed as a key technology to provide more efficient and effective tumour killing than traditional antibody therapies (figure 3).

Advances in engineered bispecific antibodies have opened a new era of antibody-based cancer treatments and more than a dozen bispecific antibodies have shown promise in clinical trials, most of them inducing the killing of target cells by engaged effector cells (table). In preclinical studies, engineered multispecific antibodies have substantially enhanced the efficacy of conventional monoclonal antibodies. However, further improvements are required with respect to antibody half-life, stability, and multiple-target selectivity, including the specific immune effector cell subset that is based on the targeted tumour microenvironment. Similar strategies used to target different RTKs should also be applied to develop antibodies that target multiple RTKs and enhance immune responses simultaneously. Ideal multispecific antibodies for cancer treatment might need to have each functional moiety targeting multiple components of the tumour microenvironment. Multiple moieties of the antigen-binding fragment or single-chain variable fragment can be combined to target tumour antigens such as overexpressed RTKs, VEGFR, and death receptors; to target T cells via CD3, CTLA-4, or PD-1; and attached to the modified fragment crystallisable region to further recruit effector

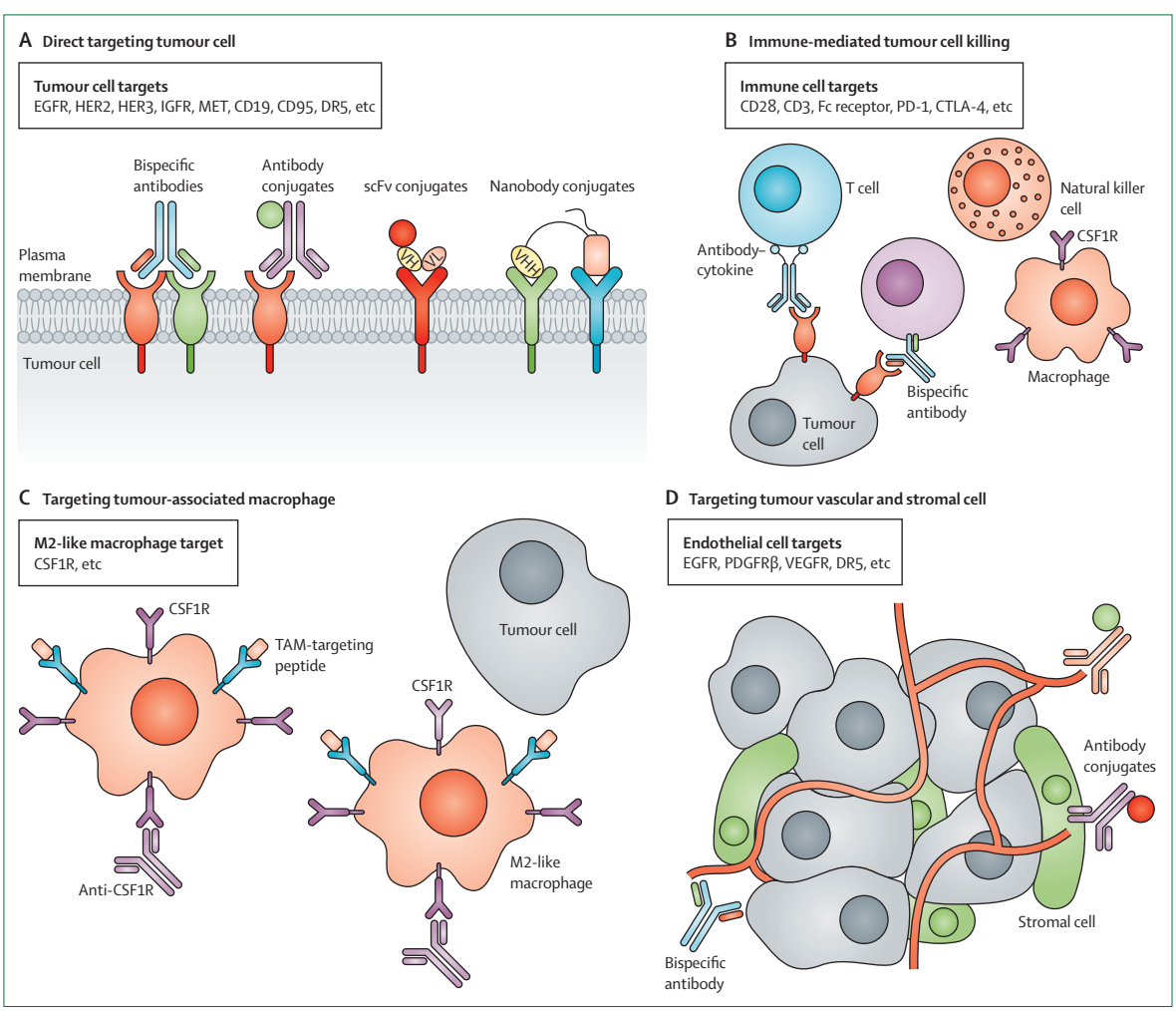


***Figure 3:*** **Strategies for targeting tumour and tumour microenvironment**
(A) Direct targeting of tumour cells by bispecific or multispecific antibodies or antibody, scFv, or nanobody conjugate binding to cell-surface receptors. (B) Modulation of immune response through bispecific antibody and antibody–cytokine recruitment of functional T cells, natural killer cells, or M1-like macrophages for immune-mediated tumour cell killing. (C) Targeting tumour-associated M2-like macrophages by an antibody or specific peptide. (D) Targeting tumour-associated endothelial cells and stromal cells by bispecific or multispecific antibodies that target cell-surface receptors. scFv=single-chain variable fragment.

### Search strategy and selection criteria

We searched PubMed and Google Scholar for references for this Review using the search terms: "bi-specific antibody", "antibody-conjugates", "nanobody", "cancer targeted therapy and resistance", "receptor tyrosine kinases", "tumor microenvironment", "angiogenesis", "cytokine", "immune cells", "tumor associated macrophage", and "clinical trials". We selected articles published in English between June 1, 1985, and Sept 1, 2015, with a focus on the past 5 years, to be included in the Review. We used the US National Institutes of Health's registry and results database of clinical studies to search for clinical trials and the international ImMunoGeneTics information system to search for information on therapeutic antibodies. The final reference list was generated on the basis of originality and relevance to the broad scope of this Review.

cells to maximise the efficacy of the treatment. Research exploring novel repertoires of tumour-selective antigens will accelerate the advancement of bispecific and multispecific antibody-based therapy for cancer. The expanding interest in this specialty will result in an array of compounds entering clinical trials in the future and will have a large effect on cancer treatment. Collection of the molecular genetic profiles of tumour cells and the tumour microenvironment, including immune-suppressive cells (tumour-associated macrophages and regulatory T cells), will help to stratify patients who are most likely to benefit from the treatment, further improving the therapeutic efficiency of antibody-based therapy in the clinic.

#### Contributors

YZ contributed to the Review outline, the scientific literature search, the creation of the table and figures, and writing of the Review. SHC contributed to the scientific literature search, creation of the table, and writing of the Review. KS contributed to the conceptual design, the Review outline, the scientific literature search, the creation of the table and figures, and the writing and finalising of the Review.

#### Declaration of interests

We declare no competing interests.

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