Introduction

With more than 20 monoclonal antibodies (mAbs) approved for therapy, and many more in clinical development, this class of molecules has become an established treatment modality for a variety of diseases. Antibody engineering is routinely applied to molecules has become an established treatment modality for a variety of diseases. Antibody engineering is routinely applied to mAbs that target only a single antigen has limitations. Blockade of multiple targets or multiple sites on one target should result in improved therapeutic efficacy. This can be achieved by combination therapy with mAbs but also other therapeutic compounds. Improved efficacy in cancer therapy has been demonstrated with combinations of mAbs targeting different receptor tyrosine kinases on cancer cells or growth factors involved in angiogenesis, or a combination of both. Furthermore, combinations of mAbs targeting two different epitopes on a single target have shown promising results. In these studies, different mAbs, with a focus on approved antibodies such as cetuximab (Erbitux®), trastuzumab (Herceptin®) and bevacizumab (Avastin®), were combined to treat solid tumors, including metastatic pancreatic cancer and breast cancer known to be dependent on expression of tyrosine kinase receptors EGFR and HER2, as well as angiogenesis induced by VEGF. However, combination therapy requires the development and approval of the individual antibodies, which involves substantial investment of resources for manufacturing, clinical studies and regulatory review. New approaches for combination therapy, therefore, include use of oligoreactive (polyclonal) antibody mixtures for the treatment of complex diseases. For example, Sym004, a mixture of two anti-EGFR antibodies, has shown promising results in preclinical studies, and is currently undergoing evaluation in a Phase 2 study (NCT01417936) in patients with...
Tables of dual targeting approaches

Table 1. Dual targeting approaches

| Targeting | Target 1 | Target 2 | Format | Indication | Reference |
|-----------|----------|----------|--------|------------|-----------|
| 2 receptors | TRAIL-R2 | LTβR | scFv-IgG, IgG-scFv | Cancer | 67 |
|           | EGFR     | IGFR | Di-diabody | Cancer | 51 |
|           | EGFR     | IGFR | diabody | Cancer | 50 |
|           | EGFR     | IGFR | scFv-IgG | Cancer | 49 |
|           | EGFR     | IGFR | PEGylated tandem Adnectin | Cancer | 52 |
|           | EGFR     | IGFR | IgG-scFv | Cancer | 9 |
|           | VEGFR2   | VEGFR3 | diabody | Cancer | 61 |
|           | VEGFR2   | VEGFR3 | Di-diabody | Cancer | 60 |
|           | VEGFR2   | PDGFRα | dAb-IgG | Cancer | 57 |
|           | PDGFRα | PDGFRβ | dAb-IgG | Cancer | 54 |
|           | HER2     | HER2 | tandem Affibody | Cancer | 151 |
|           | CD20     | CD22 | F(ab')2 | Cancer | 65 |
|           | CD20     | CD22 | IgG-scFv-DNL-Fab-IgG | Cancer | 62, 63 |
|           | FcεRI    | CD32B | kih IgG | allergic diseases | 74 |
|           | CD32B    | CD79B | DART molecule | Arthritis | 72 |
|           | MP65     | SAP-2 | tandem dAb | infectious diseases | 79 |
| 1 receptor or ligand | IGFR | IgG-scFv | Cancer | 80 |
|           | CCR5     | IgG-scFv | HIV infections | 24 |
|           | FcεRI    | tandem DARPin | allergic diseases | 81 |
|           | scorpion toxin | tandem Nanobody | envenoming | 85 |
| 2 ligands | IL-17A   | IL-23 | taFv-Fc, scFv-Fc-scFv, IgG-scFv | inflammatory diseases | 93 |
|           | IL-1α    | IL-1β | DVD-Ig | inflammatory diseases | 25, 92 |
|           | IL-12    | IL-18 | DVD-Ig | inflammatory diseases | 25 |
|           | VEGF     | osteopontin | DVD-Ig | Cancer | 89 |
|           | VEGF     | Ang-2 | CrossMab | Cancer | 30 |
|           | VEGF     | Ang-2 | CoxX-Body | Cancer | 20 |
|           | LukS-PV  | LukF-PV | HCAb-VHH | infectious diseases | 84 |
|           | PDGFRβ   | VEGF | scFv-Fc-scFv | Cancer | 100 |
|           | HER2     | VEGF | two-in-one IgG | Cancer | 26 |
|           | FcγRII   | IgE | F(ab')2 | allergic diseases | 75 |

During the past decade, dual targeting with bispecific antibodies has emerged as an alternative to combination therapy or use of mixtures. The concept of dual targeting with bispecific antibodies is based on the targeting of multiple disease-modifying molecules with one drug. From a technological and regulatory perspective, this makes development less complex because manufacturing, preclinical and clinical testing is reduced to a single, bispecific molecule. Therapy with a single dual-targeting drug rather than combinations should also be less complicated for patients.

**Dual Targeting Strategies**

Dual targeting strategies using bispecific antibodies can be divided into two types: (i) those that directly act on target structures, e.g., cell surface receptors or soluble factors (Table 1) and (ii) those that use dual targeting for delivery (retargeting) of a therapeutically active moiety, e.g., effector molecules and effector cells (Table 2). Direct actions include binding and neutralization of two ligands or two receptors, neutralization of a receptor and a ligand, activation of two receptors, activation of one receptor and neutralization of another receptor or a soluble factor, but also neutralization by binding to different epitopes of one receptor or ligand (Fig. 1A–H). Indirect actions include ADCC and CDC mediated by an Fc region, retargeting of immune effector cells through a further binding site, targeting of an effector molecule, e.g., a toxin, a cytokine or a prodrug-converting enzyme and targeting of drug-loaded nanoparticles (Fig. 1I–O). Direct and indirect actions can be combined within one molecule to further improve efficacy.

Applications of dual targeting strategies are likewise manifold, with the main indications being cancer therapy and the...
treatment of inflammatory and infectious diseases (Tables 1 and 2). Here, the same mechanisms used for combination therapy of antibodies can be targeted with bispecific antibodies. Multiple diseases mediators and signaling pathways thus can be addressed and simultaneously inhibited by the dual targeting antibody. This includes targets that act independently on different pathways, but also targets that are capable of cross-talking. Of further interest are bispecific antibodies targeting different epitopes on a disease mediator, which can lead to increased binding and enhanced neutralization.

**Bispecific Antibody Formats**

Bispecific antibodies with defined dual specificity suitable for therapeutic use must be generated through biochemical or genetic means (Fig. 2). Bispecific IgG molecules can be produced by somatic hybridization of two antibody-secreting hybridoma cells. These hybrid hybridomas (quadromas) produce within a cell two different heavy and light chains. Random assembly results in a mixture of IgG molecules, some of which are bispecific. These bispecific IgGs must be purified by two-step affinity chromatography. However, the isotypes of heavy and light chains depend on the origin of the parental antibodies, which allows little flexibility. Interestingly, however, the first bispecific antibody (catumaxomab) approved in the European Union for the treatment of malignant ascites is produced from a mouse/rat quadroma cell line, demonstrating the feasibility of this approach to generate therapeutic bispecific antibodies. Alternatively, bispecific antibodies can be generated from existing antibodies by chemical conjugation, e.g., of two IgG molecules or two Fab’ fragments, using homo- or hetero-bifunctional coupling reagents. A different chemical coupling approach is used to produce CovX-Bodies, which comprise a catalytic IgG molecule covalently coupled to reactive, bispecific peptides.

Bispecific antibodies can be also produced by genetic engineering and more than 45 different formats have been established in the past two decades (Fig. 2). A clear advantage of this approach is the greater flexibility regarding the origin of the binding site (e.g., implementing humanized or human antibodies), the composition (e.g., size, valency, isotype, presence or absence of an Fc region) and production (e.g., applying prokaryotic or eukaryotic expression systems).

A large group of recombinant bispecific antibodies are IgG-like molecules. In most of these formats, binding sites of a second specificity are fused to the N- or C-terminus of the heavy or light chain, e.g., in the form of an scFv fragment or a variable single domain, resulting in bispecific, tetravalent molecules. Bispecific molecules generated through fusion of an scFv fragment to a mAb offer great flexibility (Fig. 2). ScFv molecules have been fused to the N-terminus but also the C-terminus of the of the heavy or light chain of a mAb, generally without compromising productivity or antigen-binding activity, although issues regarding stability have recently been addressed. This group of IgG-like bispecific molecules also includes DVD-Igs, where a second V\_L and V\_H domain is fused to the heavy and light chain, respectively, of a mAb, two-in-one antibodies, where a second specificity is introduced into the natural binding site of an IgG molecule, and mAb\(^2\) molecules, where a second specificity is build into the C\(_{\text{H}3}\) domain of the Fc region. A characteristic feature of all these molecules is a symmetry caused by dimeric assembly of two identical heavy chains, an intrinsic property of these chains. A different approach is the generation of asymmetric IgG molecules. This can be achieved with the knobs-into-holes strategy. Here, amino acids at the contact site between the CH\(_3\) domains are substituted by larger or smaller residues forcing a heterodimeric assembly of heavy chains. One drawback is, however, that there is still random association with the light chains. This has been addressed by generating bispecific molecules with common light chains, or, more recently, by domain swapping between one heavy and light chain resulting in CrossMabs. Heavy chain heterodimerization was also achieved by engineering a charged CH\(_{3}\) interface to introduce an electrostatic steering effect or using the strand-exchange engineered domain technology (SEEDbody) with CH\(_{3}\) sequences composed of alternating segments from human IgA and IgG. In contrast to the bispecific IgG-like molecules, these bispecific antibodies are bivalent.

| Retargeting | Target 1 | Target 2 | Format | Indication | Reference |
|-------------|----------|----------|--------|------------|-----------|
| toxins      | CD4      | CD26     | IgG-ricin | GvHD      | 112       |
|             | CD4      | CD29     | IgG-ricin | GvHD      | 113       |
|             | CD19     | CD22     | taFv-ETA | Cancer    | 116       |
|             | CD19     | CD22     | DT-taFv  | Cancer    | 114, 115  |
|             | HER2     | EpCAM    | DT-taFv  | Cancer    | 116       |
|             | EGFR     | IL-13R   | DT-EGF-IL-13 | Cancer | 119–122  |
|             | uPAR     | IL-13R   | DT-IL-13-upA | Cancer | 123–125  |
|             | EGFR     | IL-4R    | EGF-IL-4-ETA | Cancer | 126, 127  |
| cytokines   | CD20     | HLA-DR   | F(ab)\(_2\)-IFN\(_x2b\) | Cancer | 132       |
| effector cells | CD123   | CD33     | anti-CD16 sctb | Cancer | 105       |
|             | CD19     | CD33     | anti-CD16 sctb | Cancer | 106       |
| carrier systems | CD19   | CD20     | PEGylated immunoliposomes | Cancer | 145       |
Bispecific antibodies with a molecular mass in the range of 50–100 kDa can be generated by combining the variable domains of two antibodies. For example, two scFv have been connected by a more or less flexible peptide linker in a tandem orientation (tandem scFv, taFv, tascFv), which can be extended further by additional scFv, e.g., generating bispecific or trispecific triple bodies (scb). Diabodies are heterodimeric molecules composed of the variable domains of two antibodies arranged either in the order \( V_H A - V_L B \) and \( V_H B - V_L A \) (\( V_H - V_L \) orientation) or in the order \( V_L A - V_H B \) and \( V_L B - V_H A \) (\( V_L - V_H \) orientation). The linker connecting the two domains within one chain is approximately 5 residues leading, after co-expression of the two chains within one cell, to a head-to-tail assembly and hence formation of a compact molecule with two functional binding sites. The diabody (Db) format was further stabilized by introducing interchain disulfide bonds (dsDb, DART molecules) or by generating a single-chain derivative (scDb). scDb can be converted into tetravalent molecules by reducing the middle linker, resulting in homodimerization of two chains. Small bispecific molecules have also been produced by fusing a scFv to the heavy or light chain of a Fab fragment. Furthermore, tandem scFv, diabodies and scDb have been fused to the Fc or a CH\(_3\) domain to generate tetravalent derivatives. Also, scFv can be combined with Fc or CH\(_3\) domains to generate tetravalent molecules, e.g., fusing scFvs to the N- and C-terminus of an Fc fragment, or using the knobs-into-holes approach to generate bivalent scFv-Fc or scFv-CH\(_3\) molecules. A different approach for the generation of bispecific antibodies is realized by the dock-and-lock method (DNL). Here, antibody fragments are fused to a homodimerizing docking domain (DDD) from human cAMP-dependent protein kinase A (PKA) and the anchoring domain (AD) from A-kinase anchor protein (AKAP) leading to the formation of bispecific, trivalent molecules.

Dual Targeting of Two Receptors in Cancer Therapy

Tumor development and progression often depend on growth signals mediated by receptors, which are consequently upregulated or amplified in many tumor cells. Examples include members of the EGF receptor family, i.e., EGFR, HER2, HER3 and HER4, and the IGF-1 receptor (IGF-1R), which play essential roles in regulating cell proliferation, survival, differentiation and migration. These receptors, with some exceptions, e.g., HER3 which is by its own signaling incompetent, act through multiple downstream pathways including the Ras/Raf/ERK/MAPK and the PI3K/AKT signal pathways. An acquisition of resistance to antibody therapy against a singular receptor is often associated with pathway switching between two receptors, i.e., through a compensatory upregulation and activation of the reciprocal receptor as shown for EGFR and IGF-1R, thus maintaining the malignant phenotype and leading to a relapse of the disease. Many of the established bispecific antibody formats can also be combined with additional proteins and components, e.g., drugs, toxins, enzymes and cytokines, enabling dual targeting and delivery of a fusion partner. In addition, fusion to plasma proteins such as serum albumin or albumin-binding moieties can be applied to extend the plasma half-life of bispecific antibodies.
Figure 2. Bispecific antibody formats. Variable heavy chain domains (V₇) are shown in dark blue and dark red, variable light chain domains (V_L) are shown in light blue and light red, red and blue indicating different specificities. Antibody constant domains are shown in white boxes and fusion proteins in white circles.
targeting two different receptors on a tumor cell should increase the anti-proliferative effect and help to avoid the development of resistance.

Several bispecific molecules targeting EGFR and IGF-1R have been developed, including bispecific diabodies, IgG-like tetravalent Di-diabodies, IgG-scFv fusion proteins and bispecific Adnectins™ (Table 1).9-52 Bispecific diabodies targeting EGFR and IGF-1R were generated from the anti-EGFR antibody 11F8 and the anti-IGF-1R antibody A12 and retained binding activity for the respective receptors. However, it was found that the affinity was influenced by the domain orientation and arrangement, with the V_2/V_1 orientation being superior over the V_1/V_2 orientation.50 This diabody was further converted into an IgG-like bispecific and tetravalent molecule by fusion of one of the chains to a human IgG Fc region, including the hinge region.51 This Di-diabody was produced in NS0 cells and the purified protein was shown to be capable of binding both antigens simultaneously. Inhibition of tumor cell proliferation was demonstrated in vitro, although the Di-diabody exhibited an approximately 25-fold lower inhibitory activity than the parental antibodies alone or in combination. Furthermore, the Di-diabody blocked signaling pathways stimulated by EGF and IGF-1, while the parental mAb showed inhibitory activity only for the respective pathway (Akt pathway for IGF-1R and MAPK p44/p42 for EGF). Importantly, the Di-diabody also mediated ADCC toward cells expressing either EGFR or IGF-1R, or both receptors, while the parental antibodies were only active toward tumor cells expressing their target antigen. Finally, antitumor activity was demonstrated in two xenograft mouse tumor models. In the HT29 tumor model, the bispecific tetravalent antibody was superior to treatment with the individual parental mAbs, with an activity similar to that of the combination of the two parental mAbs. The same group further developed tetravalent bispecific IgG-like molecules (scFv_1-Ig) by fusing an anti-IGF-1R scFv to the constant V_4 domain and an anti-EGFR scFv to the first C_α1 domain of the IgG heavy chain, or vice versa.49 With these constructs, similar results as for the Di-diabody were observed in vitro. The results also lead to the assumption that therapeutic response of targeting two different receptors may depend on the expression levels of the receptors and activation status of each receptor and its downstream signaling molecules.

Another IgG-like anti-EGFR x anti-IGF-1R bispecific antibody was generated by genetic fusion of a stability-engineered anti-IGF-1R scFv to the C-terminus of a chimeric aglycosylated IgG4.P/IgG1 antibody derived from an affinity-matured variant of an EGFRvIII-specific antibody isolated from a semi-synthetic phage library.9 This antibody (EI-04) demonstrated simultaneous binding of both antigens with similar affinity as the parental antibodies and concurrent blockade of ligand binding and receptor activation with IC_{50} values in the low nanomolar range. Interestingly, in a head and neck squamous cell carcinoma cell line, the bispecific antibody efficiently reduced EGFR phosphorylation, while the parental anti-EGFR antibody showed little effect. This finding indicates that the bispecific antibody is capable of inhibiting receptor pathway crosstalk in this cell line. The bispecific antibody also inhibited proliferation to a similar extent as the combination of the two monospecific antibodies. This was confirmed with a panel of tumor cell lines establishing expanded growth inhibition compared with the parental antibodies. Potent antitumor activities of EI-04 were demonstrated in tumor xenograft models. Here, the bispecific antibody administered at all dose levels was statistically more efficacious than the two parental antibodies alone using equimolar dosages. For one tumor cell line (BxPC3), EI-04 was also statistically more potent than the combination of the two parental antibodies, while with a second cell line (GEO) a similar efficacy was observed, further highlighting the complexity of target receptor biology.9

Dual targeting of EGFR and IGF-1R was further investigated for bispecific Adnectins.52 Adnectins™ represent an antibody-mimetic alternative scaffold derived from a human fibronectin domain. The bispecific Adnectins™ were generated by connecting two Adnectins™ with a flexible linker composed of ten glycine-serine repeats. Affinities of these tandem molecules were similar to those of the monospecific Adnectins™. To increase plasma half-life, the tandem Adnectins™ were coupled to a 40 kDa branched PEG chain, which resulted in a 10- to 20-fold reduction in binding, although inhibition of EGFR and IGF-1R phosphorylation in vitro was only slightly affected. Importantly, compared with the monospecific Adnectins™, the bispecific Adnectins™ were more potent in inhibiting proliferation of lung cancer cell line H292 expressing high levels of EGFR and IGF-1R. A functional benefit of having both domains within one molecule was deduced from in vivo experiments with BxPC3 xenografts. Here, the tandem Adnectin™ showed significantly better tumor growth inhibition compared with the individual monospecific Adnectins™ or a mixture of both molecules.

Dual targeting of HER2- and HER3-expressing tumor cells was described for a bispecific molecule generated by fusing scFvs directed against HER2 and HER3 to the N- and C-terminus of human serum albumin (scFv-HSA-scFv).53 This molecule (MM-111) combines targeting of HER2-overexpressing tumor cells with potent inhibition of ligand-induced phosphorylation of HER3 with IC_{50} values in the sub-nanomolar range. Computational physicochemical modeling was applied to optimize the monovalent binding affinities to increase potency and specificity for tumor cells. MM-111 is currently undergoing evaluation in three Phase 1 studies in patients with advanced HER2-amplified cancers (NCT00911898, NCT01097460 and NCT01304784).

Simultaneous receptor blockage with bispecific antibodies was also applied for platelet-derived growth factor receptor α (PDGFRα) and β (PDGFRβ).54 These receptors are activated by members of the PDGF family and are capable of forming homo- as well as heterodimeric receptor complexes. PDGFRs have been identified on a number of tumor types and are involved in stimulation of tumor cells, but also angiogenesis.55,56 Bispecific IgG-like antibodies against the two PDGF receptors were generated by fusion of an anti-mouse PDGFRα single variable domain (sVD) either to the N-terminus of the light chain (sVD-IgG) or the C-terminus of the heavy chain (IgG-sVD) of an anti-mouse PDGFRβ IgG (see also Fig. 2). Simultaneous binding of antigens
was demonstrated for the bispecific antibodies and both antibodies were capable of blocking binding of the ligands PDFG-AA and PDFG-BB to its receptors, which resulted in inhibition of ligand-mediated receptor phosphorylation. In these assays, the IgG-sVD fusion protein showed better effects than the sVD-IgG fusion protein indicating that the position of adding a second binding site has a direct influence on bioactivity.

Another sVD-IgG construct was generated by fusing the anti-mouse PDGFRα sVD to the N-terminus of the light chain of an anti-mouse VEGFR2 IgG. Thus, this bispecific antibody targets another receptor involved in tumor angiogenesis. The bispecific antibody was able to recognize both receptors simultaneously and to inhibit PDGF and VEGF-induced stimulation of murine endothelial cells. Neutralization of stimulating receptors of tumor endothelial cells was also studied with a bispecific diabody directed against VEGFR2 (KDR) and VEGFR3 (Flt-4) or a Di-diabody (diabody-C₃,3 fusion protein) directed against VEGFR1 (Flt-1) and VEGFR2. For these constructs, inhibition of ligand binding and VEGF-induced cell migration was described.

Based on results from a Phase 2 study of combination therapy with mAbs for the treatment of B cell lymphoma combining an anti-CD20 antibody (rituximab) with an anti-CD22 antibody (epratuzumab), it was postulated that bispecific antibodies should also be applicable for the treatment of hematologic malignancies. A tetravalent IgG-like bispecific antibody generated by fusing an anti-CD22 scFv from epratuzumab to the C-terminus of the heavy chain of an anti-CD20 IgG veltuzumab showed improved inhibition of cell proliferation as well as ADCC against Daudi cells in vitro. In vivo, beneficial therapeutic effects of the bispecific antibody were, however, seen only at the highest dose. Of interest was the finding that the bispecific antibody induces translocation and accumulation of B cell receptors (BCR) in lipid rafts, presumably due to a stronger interaction of CD22 with the BCR, which was thought to lead to increased growth inhibition and apoptosis. This approach was extended by the same group using a hexavalent IgF-G fab molecule produced by the dock-and-lock method. Potent inhibition of cell proliferation was also observed for this bispecific antibody. Again, antitumor activity was similar to that observed for a combination of the parental antibody. In another study, a CD20 x CD22-specific bispecific F(ab′)₂ molecule generated by chemical conjugation showed significantly improved antitumor effects compared with treatment of the parental antibodies rituximab (CD20) and HB22.7 (CD22) alone or in combination. All the studies established that the bispecific antibodies induce a stronger p38 phosphorylation, which might be responsible for the increased cytotoxic effects observed in vitro. Furthermore, it was discussed that the bispecific antibody prevents rapid internalization of CD22 through binding to non-internalizing CD20, thus affecting downstream signaling.

The studies with anti-CD20 and anti-CD22 bispecific antibodies demonstrated that targeting of relevant cell surface receptors can lead to an efficient activation of signaling pathways and increased anti-proliferative and cytotoxic effects without utilizing ADCC or CDC. Induction of cell death through apoptosis is also known for members of the death receptor family, e.g., TRAIL receptors, which has led to the development of agonistic antibodies for cancer therapy. The applicability of bispecific antibodies as agonists of death receptors was investigated with IgG-scFv fusion proteins targeting TRAIL receptor 2 (TRAIL-R2, DR5) and lymphotoxin-β receptor (LTBR), which are co-expressed on the surface of a variety of cell lines from tumors of epithelial origin. Bispecific IgG-scFvs were produced by fusing a stability-improved disulfide-linked anti-LTBR scFv either to the N- or C-terminus of the anti-TRAIL-R2 IgG heavy chain. An enhanced antitumor activity was observed for a subset of tumor cell lines in vitro relative to the combination of parental antibodies. In vivo, superior tumor growth inhibition was observed for MDA-MB-231 xenografts and a bispecific antibody with the scFv fused to the heavy chain C-terminus, while for another cell line (WiDr) effects were similar to that of the combination therapy, in accordance with the observed in vitro activity.

**Dual Targeting of Two Receptors for the Treatment of Inflammatory Diseases**

Self-perpetuating B lymphocytes have been identified as contributors to the development and progression of inflammatory and autoimmune rheumatic diseases. Therefore, B cell depletion or inactivation represents a viable therapeutic approach, as shown by the use of rituximab in the treatment of rheumatoid arthritis. B cell activation is induced by antigen binding to the B cell receptor (BCR) and regulated by a negative feedback loop mediated by the inhibitory Fcγ receptor IIb (FcyRIIb; CD32B) induced by a close contact between the two receptors upon binding of antigen to the BCR and soluble immunoglobulins.

To recapitulate this antigen-driven proximity of the activating and inhibitory receptors, a bispecific DART molecule directed against CD79b, which is a signaling-competent molecule of the BCR and CD32B was generated. Simultaneous binding to both receptors resulted in inhibition of B cell proliferation and secretion of immunoglobulins and reduced disease severity of collagen-induced arthritis in mice, indicating that bispecific antibodies as activation-dependent inhibitors are useful for the treatment of autoimmunity. A similar approach was applied for the treatment of allergic diseases. Crosslinking of the inhibitory CD32B receptor to the FceRI delivers a dominant-negative signal that efficiently suppresses all activating signals of the FcεRI receptor expressed by mast cells and basophils. This was shown previously with a dimeric dual targeting Fcy-Fce functional fusion protein. A bispecific IgG recognizing FcεRI and CD32B was generated using a knobs-into-holes approach. This antibody inhibited the activation of mast cells in vitro and in a passive cutaneous anaphylaxis (PCA) model. Furthermore, coaggregation of FceRI and CD32B and inhibition of histamine release was also achieved using a bispecific F(ab′)₂ molecule directed against IgE and FcγRII.
Dual Targeting of Two Receptors for the Treatment of Infectious Diseases

Many pathogens interact through specific adhesion molecules with receptors on host cell or use surface-exposed enzymes to facilitate infection. Interference with these molecules can, therefore, be employed to inhibit infectivity and pathogen spreading, e.g., by passive immunization with pathogen-reactive antibodies or serum preparations. Treatment with these reagents is especially appropriate in patients with an impaired immune system or who have become refractory to treatment with anti-microbial drugs. Here, dual targeting can improve an anti-microbial response.

Candidiasis, a fungal infection caused by the opportunistic, mucosal pathogen *Candida albicans*, can develop into a life-threading disease in severely immuno-compromised individuals. Mannoprotein MP65 and the secreted protease Sap2 represent important virulent factors. A bispecific antibody against these two targets was generated by fusing two human domain antibodies (dAbs) in tandem, and it was shown to inhibit adherence to vaginal epithelium and to protect against experimental vaginal candidiasis. Interestingly, an accelerated fungal clearance from vagina was observed for the bispecific dAb molecule compared with the monospecific molecules.

**Dual Targeting of Different Epitopes of One Receptor or Ligand**

MAbs against cell surface receptors can exert their inhibitory potential through different mechanisms, including competitive and allosteric blockage of ligand binding. For IGF-1R, which can be activated by two ligands, IGF-1 and IGF-2, it was shown that antibodies recognizing different epitopes of the receptor exhibit an increased neutralizing potential in vitro and in vivo. Two of these antibodies, one being a competitive and the other being an allosteric inhibitor of ligand binding, were combined into tetravalent, bispecific IgG-like molecules by fusing scFv fragments either to the N- or C-terminus of the IgG heavy chain. A comparison of various molecules revealed that only one of these antibodies, BIIB4-5scFv, was capable of binding with all of its arms to IGF-1R and showed in vitro an inhibitory activity comparable to that of the combination of the two mAbs. Using established xenograft models of SJSA-1 tumors, an increased antitumor activity was observed for BIIB4-5scFv compared with the single mAbs or the bispecific antibody 5scFv-BIIB4 that had the scFv fused to the N-terminus of the heavy chain. However, using other xenograft models, the superior activity of BIIB4-5scFv could not be repeated or was not statistically significant. One reason for this finding might be that these tumor cells depend on multiple growth and survival signals and that inhibition of IGF-1R signaling is not sufficient to suppress tumor growth.

Dual targeting of different epitopes of one receptor was also applied for the treatment of HIV infections using stability-improved IgG-scFv fusion proteins fusing scFv either to the N- or C-terminus of the heavy or light chain, respectively. These bispecific antibodies block two alternative docking sites of the CCR5 co-receptor of HIV. To improve the stability of the fusion proteins, the scFv fragment was further stabilized by introducing a disulfide-bond between the V\_H and V\_L domain or by increasing the linker length connecting the V\_H and V\_L domain from 15 residues to 30 residues, which drastically reduced the aggregation tendency. While binding to CCR5-expressing CHO cells was increased only 1.3- to 1.7-fold, an 18- to 57-fold increased antiviral activity was observed for the different variants compared with the parental antibodies. Importantly, the bispecific antibodies were also capable of inhibiting virus strains resistant to the treatment with the single parental antibodies. These findings demonstrate that dual targeting with bispecific antibodies can improve antiviral activity and can even overcome resistance to treatment with monospecific mAbs.

Application of the emerging class of scaffold proteins for dual targeting was shown for a bispecific designed ankyrin repeat protein (DARPin) targeting two epitopes of the high-affinity Fcε receptor (FcεRI). Aggregation of this receptor by allergen-induced cross-linking of bound IgE induces degranulation of basophils and mast cells and can induce allergy symptoms by the release of proinflammatory molecules. Treatment options include anti-IgE antibodies, which prevent binding of IgE to FcεRI. Omalizumab, a humanized antibody directed against the Fc part of IgE is approved for the therapy of severe asthma. Therapy should also be possible with antibodies directed against the FcεRI; however, it is essential that binding does not induce cross-linking and activation of the receptor. Eggel and coworkers addressed this problem by generating a bispecific DARPin targeting the two IgE-binding sites of FcεRI located in the extracellular FcεRIα chain. Bispecific DARPins were generated connecting two DARPin sites by a flexible peptide linker. One of these molecules, DARPin 30/85, showed a strongly increased affinity compared with the parental DARPin and simultaneously blocked both epitopes of the receptor without forming receptor aggregates. However, it was shown that anti-DARPin antibodies can induce cross-linking and degranulation, which might limit therapeutic applications.

Dual targeting can also be applied to efficiently neutralize toxins, e.g., from bacteria, fungi, plants or animals. Feasibility was demonstrated, for example, with a bispecific heavy chain antibody generated by fusing a VH\_H domain to the C-terminus of a llama heavy chain antibody. This tetravalent, bispecific HCAb targets two subunits (Lυc\_PV and Lυc-F\_PV) of *Staphylococcus leukotoxin Panton-Valentine leukokidin* (PVL), which is associated with human pyogenic necrotizing skin infections and more severe septic infections. The antibody bound both antigens simultaneously and inhibited formation of new pores by preventing binding of the toxin components to membranes of peripheral mononuclear cells, monocytes and lymphocytes. However, the antibody had no effect on already formed pores. Effectiveness was further demonstrated in vivo in a non-infectious PVL-induced rabbit endophthalmitis model. Compared with equimolar amounts of the bivalent, monospecific HCAbs, inhibition of inflammatory reactions and tissue destruction was more efficient with the tetravalent, bispecific molecule.
In another study, a bispecific Nanobody was developed for the treatment of *Androctonus australis hector* (Aah) scorpion envenoming.35 The venom contains three small toxins with a molecular mass of 7 kDa that rapidly distribute in the blood and tissues. Currently, intoxicated people are treated with a polyclonal equine F(ab')2-based antivenom. However, these fragments reach the tissue much slower than the toxin, which necessitates high doses applied intravenously.86 The bispecific Nanobody NbF12-10 directed against AahI and AaHII, possessing a size of only 29 kDa, was highly potent in protecting mice from lethal doses of the scorpion venom when administered subcutaneously, in contrast to treatment with the plasma antivenom serum-derived F(ab')2, which was ineffective under these conditions.

**Dual Targeting of Two Ligands in Cancer Therapy**

The growth of solid tumors depends on neovascularization promoted by vascular growth factors.87 These angiogenic factors induce endothelial cell proliferation and migration, extracellular matrix remodeling, increased vascular permeability and survival of the newly formed blood vessels.88 Besides VEGF-A, several other proteins with angiogenic activity have been identified, including angiopoietin-2 (Ang-2) and osteopontin. Neutralization of these factors with mAbs interferes with the formation of novel blood vessels, as shown for bevacizumab, an anti-VEGF antibody approved for the treatment of metastatic colorectal cancer and various other solid tumors. Simultaneous neutralization of different angiogenic molecules should further improve the anti-angiogenic activity. This was demonstrated for bispecific DVD-Igs generated by fusing either the variable domains of an anti-osteopontin antibody (hu1A12) to the N-terminus of the heavy and light chains of bevacizumab (VEGF/OPN-BsAb) or the other way round (OPN/VEGF-BsAb).89 Both antibodies showed similar binding behavior as the parental antibodies and VEGF/OPN-BsAb was chosen for further analysis. The bispecific antibody efficiently inhibited growth of endothelial cells in vitro, reduced strongly the micro-vessel density (MVD) in a hepatocellular carcinoma model (HCCLM3) and potently suppressed the growth of primary tumors and the formation of spontaneous lung metastases, suggesting that this approach has potential in treating metastatic cancers. In all these experiments, the activity was increased compared with treatment with the bevacizumab and hu1A12 alone, but similar to treatment with a combination of both parental antibodies.

In another study, the CrossMab format was applied to generate bivalent, bispecific IgG molecules directed against VEGF-A and Ang-2.90 One of these antibodies, CrossMabCH1-CL, showed favorable stability properties and was capable of simultaneously binding to both antigens with comparable affinities as the parental antibodies bevacizumab and LC06. Inhibition of Colo205 tumors by the CrossMab was similar to treatment with a combination of bevacizumab and LC06 and more effective that single antibody treatment. Furthermore, similar results were observed for inhibition of VEGF-induced corneal angiogenesis, emphasizing the versatility of dual targeting strategies.

**Dual Targeting of Two Ligands in the Treatment of Inflammatory and Autoimmune Diseases**

Multiple disease modulators play an essential role in the pathogenesis of inflammatory and autoimmune diseases having either a redundant activity, i.e., acting on the same signaling cascade, or acting on two or more independent pathways. Simultaneous inhibition of different disease modulators should therefore be beneficial for therapy, although studies from combination therapies, e.g., with etanercept (Enbrel90) and abatacept (Ocrevus91), did not reveal improved efficacy but an increase in infectious complications,92 underlining the adage that targets have to be carefully selected.

Dual targeting of disease-modulating cytokines was evaluated with various bispecific antibodies. A tetravalent, bispecific DVD-Ig that simultaneously bound and neutralized IL-12 and IL-18 was generated.93 This antibody bound to the two cytokines with similar affinities as the parental antibodies and efficiently inhibited IL-12 and IL-18-induced IFNα release in vitro. Therapeutic efficacy was demonstrated for *Staphylococcus aureus* dried cell (SAC)-induced IFNα production in SCID mice. Here, the bispecific antibody almost completely abrogated IFNα production and was as efficient as a combination of the two parental antibody and more potent than the mAbs alone. In the same study, a bispecific DVD-Ig directed against mouse IL-1α and IL-1β was generated. This bispecific antibody inhibited both pro-inflammatory cytokines with IC50 in the low nanomolar range. However, compared with the parental antibodies, the neutralizing activity was 10-fold reduced for IL-1α and 2-fold reduced for IL-1β. Nevertheless, in a collagen-induced arthritis (CIA) model, the bispecific antibody strongly inhibited disease progression, similar to a combination of the parental antibodies, as well as potency-matching antibodies. It was further found that the orientation and the linker lengths influenced stability, aggregation tendency, as well as binding and affinity. Thus, a DVD-Ig directed against human IL-1α and IL-1β was generated and further optimized by varying the domain orientation and the length of the linkers connecting the variable domains.92

Dual targeting and neutralization was further demonstrated for bispecific antibodies directed against IL-17A and IL-23.93 IL-23 is a cytokine that stimulates the differentiation and
regulation of Th17 helper T cells, which in turn produce several pro-inflammatory cytokines such as IL-17. Th17 cells are thus dominating cell types associated with autoimmune disorders, e.g., rheumatoid arthritis, with IL-23 and IL-17 being important disease-promoting factors. Stable scFv molecules directed against IL-17A and IL-23 were selected from a phage library and employed for the generation of various tetravalent, bispecific antibodies: a tandem scFv-Fc fusion protein (tascFv-Fc), a scFv-Fc scFv fusion protein and an IgG-scFv with the scFv fused to the C-terminus of the heavy chain. An increased thermal stability was determined for the IgG-scFv fusion protein compared with the two other formats. All bispecific variants exhibited 6- to 8-fold decreased affinity for the two cytokines, but were capable of binding both cytokines simultaneously. Compared with soluble IL-23 receptor, an up to 10-fold increased neutralization activity was observed in a murine splenocyte assay. Pharmacokinetic studies demonstrated that the tascFv-Fc and IgG-scFv fusion proteins had superior serum half-lives.

**Dual Targeting of a Receptor and a Ligand in Cancer Therapy**

Signal transduction is induced by binding of a ligand to a receptor. Inhibition of growth and differentiation-promoting signals can be achieved by inhibiting either the receptor or the ligand, as illustrated by the various examples discussed. This offers also an approach for dual targeting by simultaneously inhibiting a receptor and a ligand for the same or another receptor. Feasibility was shown for dual inhibition of HER2 and VEGF with a combination of trastuzumab and VEGF-trap, thus, combining targeting of tumor cells with an anti-angiogenic approach. This combination is of special interest because HER2-overexpressing tumor cells have been shown to secret elevated levels of VEGF. A bispecific antibody against HER2 and VEGF was recently generated applying the “two-in-one” antibody strategy. In this study, a second specificity for VEGF was grafted into the binding site of the anti-HER2 trastuzumab. This reduced the affinities for both antigens. Affinity improved variants were therefore selected from phage libraries. One of these variants, bH1-44, inhibited the growth of HUVECs and BT474 tumor cells in vitro to a similar extend as bevacizumab or trastuzumab, respectively, and also showed potent anti-tumor activity in two xenograft tumor models, which was similar or even better than a combination of bevacizumab and trastuzumab.

Dual targeting of a receptor and a ligand can also be applied for inhibition of different signals involved in angiogenesis. For example, beneficial effects of co-inhibition of PDGF- and VEGF-mediated signaling were shown for a combination therapy with receptor-specific small molecule tyrosine kinase inhibitors or with mAbs directed against PDGFRβ and VEGFR. In another study, a bispecific antibody was applied to simultaneously inhibit PDGFRβ and VEGF-A. The antibody was generated by fusing different scFvs to the N-terminus and C-terminus of an Fc fragment (scFv-Fc-scFv), resulting in a tetravalent, bispecific molecule with the two chains covalently linked by disulfide bonds of the hinge region. This bispecific antibody inhibited proliferation of HUVECs, as well as human brain vascular pericytes (HBVPs), with IC₅₀ values in the picomolar range. In a co-culture sprouting assay mimicking the in vivo generation of blood vessels from endothelial cells and mesenchymal stem cells (MSC), the bispecific antibody inhibited endothelial sprouting and pericyte dissociation from endothelial cells. Although not cross-reactive with mouse VEGF and PDGFRB, inhibition of tumor growth and reduction of the micro-vessel density (MVD) were observed in a xenograft mouse model, similar to treatment with bevacizumab.

**Dual Retargeting of Effector Cells**

Bispecific antibodies have been widely used for the retargeting of immune effector cells to tumor cells. In this case, one binding site is directed against a tumor-associated antigen and the second antigen against a trigger molecule on the effector cells. Thus, bispecific antibodies have been employed for the retargeting of T cells by binding to CD3, which is part of the T cell receptor complex, or of natural killer (NK) cells by binding to the FcγRIII (CD16). Blinatumomab, a recombinant bispecific tandem scFv molecule (bispecific T cell engager, BiTE) directed against CD3 and CD19 is in clinical trials and has shown promising results in Phase 1 and 2 studies in non-Hodgkin lymphoma and ALL patients. Strong lysis of double-positive tumor targets was observed in a murine splenocyte assay. Pharmacokinetic studies demonstrated that the tascFv-Fc and IgG-scFv fusion proteins had superior serum half-lives.

**Dual Retargeting of Toxins**

Extensive work has been performed for dual retargeting of toxins. Targeted toxins are generated by coupling or fusion of a toxin, e.g., ricin or others derived from bacteria, fungi or plants, to antibodies or other ligands. In immunotoxins, the cell-binding moiety of the toxin is replaced by the antibody, thus redirecting the toxin to a tumor cell and leading to target-mediated internalization of the fusion protein. The toxin fragment contains a translocation domain necessary for release of the catalytic
domain into the cytosol. Feasibility of this approach is demonstrated with denileukin diftitox (Ontak®), which is approved for the treatment of cutaneous T cell lymphoma.\textsuperscript{109} Denileukin diftitox is a fusion protein comprising a Diphtheria toxin fragment (DAB$_{189}$) fused to IL-2 as ligand for IL-2R expressed on activated T cells. Various antibody-toxin fusion proteins have been generated, e.g., by fusion of an scFv or disulfide-stabilized Fv fragment to a toxin, for the treatment of hematologic and solid tumors and several have already entered clinical trials.\textsuperscript{108} Despite the clinical responses observed, these studies also revealed various challenges of this approach, including nonspecific toxicities, stability and production issues and immunogenicity. The latter has been addressed by use of humanized or human antibody fragments and by generating deimmunized versions of the toxin, e.g., as shown for Pseudomonas exotoxin A.\textsuperscript{110}

Dual targeting strategies have been developed to improve efficacy of immunotoxins (Table 2).\textsuperscript{111} Initial studies applied bispecific IgG molecules conjugated with a toxin. Thus, an immunotoxin was generated using a quadroma-derived bispecific IgG directed against CD4 and CD26 coupled to a ricin holotoxin for the elimination of activated T cells, e.g., to prevent graft-vs.-host disease and autoimmunity.\textsuperscript{112} The bispecific antibody caused a drastically increased internalization and 2- to 3-fold increased cytotoxicity compared with the parental antibodies. Similar results were described for an IgG-immunotoxin directed against CD4 and CD29 developed for targeting and depletion of restricted T-cell subsets.\textsuperscript{113}

More recently, the concept was evaluated with recombinant bispecific immunotoxins generated either by fusing a tandem scFv to the N-terminus of an exotoxin A fragment or to the C-terminus of a Diphtheria toxin fragment, respectively. These bacterially produced bispecific immunotoxins were directed against CD19 and CD22 and demonstrated an improved efficacy against mouse xenograft models of B cell malignancies and metastases. Because of their dual specificity, it was discussed that these types of immunotoxins broaden the reactivity against most lymphomas and B cell leukemia, as shown for the Diphtheria immunotoxin.\textsuperscript{114}

Problems arising from an aggregation tendency were addressed by domain swapping within the tandem scFv and use of an aggregation-reducing linker, which also improved therapeutic efficacy in animal models.\textsuperscript{115} Furthermore, a deimmunized version of exotoxin A was used to generate a bispecific immunotoxin directed against CD19 and CD22, which strongly reduced the production of a neutralizing antitoxin immune response while maintaining its cytotoxic activity. This should allow multiple drug treatments and result in improved therapeutic activity.\textsuperscript{116}

In another study, a bispecific immunotoxin comprising an anti-HER2 scFv fused to a Diphtheria toxin-anti-EpCAM immunotoxin was generated for the treatment of solid tumors overexpressing HER2 and EpCAM.\textsuperscript{117} Compared with monospecific immunotoxins, increased cytotoxicity toward tumor cells expressing both antigens was observed in vitro and in xenograft tumor models.

As an alternative to the use of antibody fragments, various bispecific ligand-directed toxins (BLT) were generated using natural ligands such as growth factors and cytokines for targeting. For example, dual targeted toxins were generated fusing IL-13 and EGF with Diphtheria toxin. In other studies, IL-13 was combined with uPA\textsuperscript{124,125} IL-4 with EGF,\textsuperscript{126,127} or EGF with uPA,\textsuperscript{128} fused either to Diphtheria toxin or Pseudomonas exotoxin A (Table 2). Superior activities were seen with this BLTs, e.g., after intratumor injections into subcutaneous xenograft tumors. The interaction of the ligands with receptors on normal cells was reduced by applying a ToxBloc method. This involves a bolus IP injection of recombinant bispecific fusion protein without toxin prior to application of the immunotoxin, which increased the MTD by 15-fold, as shown for an Diphtheria toxin-EGF-IL-13 fusion protein.\textsuperscript{122} These studies illustrate the great flexibility of this system.

### Dual Retargeting of Cytokines

A growing number of immunostimulatory and apoptosis-inducing cytokines are being developed for tumor therapy, including interleukins, interferons, growth factors and TNF family members. Interleukin-2 (IL-2) was approved for the treatment of RCC and metastatic melanoma and TNF is used for the treatment of sarcoma and melanoma by isolated limb perfusion in combination with chemotherapy.\textsuperscript{129} Furthermore, GM-CSF and its PEGylated derivative are used for the treatment of neutropenia, e.g., during myelosuppressive chemotherapy. Targeted delivery of these cytokines, e.g., by fusion to an antibody fragment, can result in enhanced therapeutic efficacy by increased tumor localization.\textsuperscript{130} Examples include antibodies and antibody fragments fused to IL-2, IL-10, IL-12, IL-15, IFNα, IFNβ, IFNγ, TNF, TRAIL and FasL. Several of these fusion proteins have already entered clinical Phase I studies.\textsuperscript{131} Dual targeting was applied to an IFNα2b immunocytokine (20-C2-2b) by combining two copies of IFNα2b with a humanized and stabilized F(ab)$_2$ directed against HLA-DR and a humanized anti-CD20 IgG using the dock-and-lock method.\textsuperscript{132} An increased cytotoxicity against various lymphoma and myeloma cell lines was observed in vitro compared with a monospecific antibody-IFNα fusion protein targeting only CD20 or a mixture of the parental antibodies. Furthermore, the bispecific immunocytokine was more potent in killing tumor cells expressing CD20 and HLA-DR than the monospecific immunocytokine, indicating that binding to both antigens enhances the cytotoxic effect of the cytokine. This study also showed that sensitivity to the cytokine, as well as expression and density of the target antigens, determine responsiveness toward the immunocytokine.

### Dual Targeting of Liposomes and Nanoparticles

Nanoparticles are versatile carrier systems for the delivery of drugs.\textsuperscript{133,134} Use of liposomes attracted early interest, which resulted in the approval of various liposomal drugs, e.g., for cancer therapy and treatment of fungal infections.\textsuperscript{135} Carrier systems are able to protect the drug from rapid elimination and degradation. Furthermore, long-circulating particles such as PEGylated liposomes, utilize the enhanced permeability and retention (EPR) effect to passively accumulate in the tumor...
tissue.\textsuperscript{136} PEGylated liposomal doxorubicin (Doxil\textsuperscript{®}, Caelyx\textsuperscript{®}) is approved for the treatment of ovarian cancer, multiple myeloma and AIDS-related Kaposis sarcoma.\textsuperscript{137} Delivery of nanocarriers to tumor cells or other structures can be achieved by insertion of ligands into the particle surface, thereby enabling active targeting. For example, sterically-stabilized immunoliposomes are generated by coupling antibody or antibody fragments to PEG chains inserted into the lipid bilayer.\textsuperscript{138,139} Internalization of drug-loaded nanocarriers has been identified as an important prerequisite for efficient drug delivery.\textsuperscript{140,141} This can be achieved using ligands or antibodies binding to rapidly internalizing receptors. Furthermore, intracellular release of the encapsulated drug from the endosomal compartment has been found to be equally important. Strategies to facilitate endosomal release include the implementation of pH-sensitive mechanisms.\textsuperscript{142}

Preferably, immunoliposomes are generated using antibody fragments devoid of an Fc region to avoid uptake by cells of the reticuloendothelial system.\textsuperscript{143} A first immunoliposomal formulation of doxorubicin (MM-302) comprising an anti-HER2 scFv for targeting of HER2-positive cancers is currently in a Phase 1 trial to evaluate safety and pharmacokinetic properties in patients with advanced breast cancer (NCT01304797),\textsuperscript{144} illustrating that even complex therapeutics composed of lipids, antibodies and drugs can be formulated for clinical use.

Nanoparticles should also benefit from targeting different antigens on a cancer cell, thus improving binding, selectivity and drug delivery. Dual targeting of nanocarriers with antibodies was demonstrated for PEGylated liposomes conjugated with monoclonal IgG directed against CD99 and CD20.\textsuperscript{145} These dual targeting immunoliposomes were generated by inserting equal amounts of anti-CD99 and anti-CD20 IgG coupled to maleimide-PEG-DSE. In this study, additive effects were observed for binding and internalization of a combination of anti-CD99 and anti-CD20 immunoliposomes, similar to effects seen with dual targeting immunoliposomes at equal antibody and liposome concentrations, which translated into a tendency to somewhat lower IC\textsubscript{50} values in in vitro cytotoxicity assays with doxorubicin-loaded liposomes.

Dual targeting of nanocarriers can also be achieved using natural ligands and synthetic peptides. This was shown, for example, with nanoparticles conjugated with folate and an anti-EGFR antibody,\textsuperscript{146} or combining an α\textsubscript{i} integrin-binding RGD peptide with ligands such as transferrin,\textsuperscript{147} neurophilin,\textsuperscript{148} galectin-1,\textsuperscript{149} or an NGR peptide recognizing CD13.\textsuperscript{150} Results from one study with paclitaxel-loaded liposomes targeted to tumor cells and the tumor vasculature support the concept that dual targeting of different cell types improves the antitumor activity of drug-loaded nanocarriers.\textsuperscript{151}

Conclusions

During the past decade a plethora of novel bispecific antibody formats have been developed and used for dual targeting strategies. Although a strong focus is on development of treatments of cancer and inflammatory diseases, additional applications include treatment of other disorders such as infectious and allergic diseases. Results from these studies demonstrate that, in general, bispecific antibodies outperform treatment with monospecific antibodies, but are similar to the combination of parental antibodies, although for some indications increased potency was described. The examples clearly show that bispecific antibodies targeting different disease modulators are capable of improving standard therapy. There are still issues to be addressed, e.g., manufacturing, stability and pharmacokinetic properties of the bispecific antibodies. Nevertheless, the growing interest in this field and the potential of bispecific antibodies for dual targeting strategies suggest that these molecules will enter clinical study in increasing numbers in the near future.

References

1. Reichert JM. Antibody-based therapeutics to watch in 2011. mAbs 2011; 3:76-99; PMID:21051951; http://dx.doi.org/10.4161/mabs.3.1.13895.
2. Nelson AL, Dhimola E, Reichert JM. Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov 2010; 9:767-74; PMID:20811384; http://dx.doi.org/10.1038/nrd3229.
3. Presta LG. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. Adv Drug Deliv Rev 2006; 58:640-56; PMID:16904789; http://dx.doi.org/10.1016/j.addr.2005.08.004.
4. Doherty AD, O’Shaughnessy JA, Haas J, et al. Recombinant proteins in rheumatology—recent advances. N Biotechnol 2011; 28:502-10; PMID:21473939; http://dx.doi.org/10.1016/j.nbt.2011.03.019.
5. Jones HE, Gee JMW, Hutcheson IR, Knowlden JM, Barrow D, Nicholson RI. Growth factor receptor interplay and resistance in cancer. Endocr Relat Cancer 2006; 13:45-51; PMID:17295958; http://dx.doi.org/10.1677/erc.1.01275.
6. van der Veeken J, Oliveira S, Schifflers RM, Storm G, van Berge En Henegouwen PM, Roovers RC. Crosstalk between epidermal growth factor receptor- and insulin-like growth factor-1 receptor signaling: implications for cancer therapy. Curr Cancer Drug Targets 2009; 9:748-60; PMID:19754559; http://dx.doi.org/10.2174/156800909789271495.
7. Mognillo F, Lee HY. Resistance to epidermal growth factor receptor-targeted therapy. Drug Resist Updat 2005; 8:298-310; PMID:16176217; http://dx.doi.org/10.1016/j.drup.2005.08.004.
8. Dong J, Sereno A, Avrilzian D, Langlely E, Miller BR, Snyder WB, et al. A stable IgG-like bispecific antibody targeting the epidermal growth factor receptor and the type I insulin-like growth factor receptor demonstrates superior anti-tumor activity. mAbs 2011; 3:273-88; PMID:21359933; http://dx.doi.org/10.4161/mabs.3.3.15188.
9. Demarest SJ, Hariharan K, Dong J. Emerging antibody combinations in oncology. mAbs 2011; 3:338-51; PMID:21697653; http://dx.doi.org/10.4161/mabs.3.4.16615.
10. Haueum JS. Recombinant polyclonal antibodies: the next generation of antibody therapeutics? Drug Discov Today 2006; 11:655-60; PMID:16793535; http://dx.doi.org/10.1016/j.drudis.2006.05.009.
11. Koeboe K, Steinhaa L, Sønderberg JN, Kjer I, Jacobsen HJ, Meijer PJ, et al. Rational identification of an optimal antibody mixture for targeting the epidermal growth factor receptor. mAbs 2011; 3; In press; PMID:22123060.
12. Pedersen MW, Jacobsen HJ, Koeboe K, Hey A, Pyke C, Haureum JS, et al. Sym004: a novel synergistic anti-epidermal growth factor receptor antibody mixture with superior anticancer efficacy. Cancer Res 2010; 70:588-97; PMID:20068188; http://dx.doi.org/10.1158/0008-5472.CAN-09-1417.
13. Skærved NJ, Jacobsen HJ, Pedersen MW, Jensen PF, Sen JW, Jørgensen TK, et al. Preclinical pharmacokinetics and safety of Sym004: a synergistic antibody mixture directed against epidermal growth factor receptor. Clin Cancer Res 2011; 17:5962-72; PMID:21825941; http://dx.doi.org/10.1158/1078-0432.CCR-11-1209.
14. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol 2010; 10:301-16; PMID:20414204; http://dx.doi.org/10.1038/nri2761.
15. Muller D, Kontermann RE. Bispecific antibodies for cancer immunotherapy: Current perspectives. BioDrugs 2010; 24:89-98; PMID:20199124; http://dx.doi.org/10.2165/11530960-00000000-0000.0000.
16. Kontermann RE. Bispecific antibodies. Springer 2011; ISBN 978-3-642-20909-3.
29. Doppalapudi VR, Huang J, Liu D, Jun P, Liu B, Li L, et al. Chemical generation of bispecific antibodies. Proc Natl Acad Sci USA 2010; 107:22611-6; PMID:21149738.

30. Coloma MJ, Morrison SL. Design and production of novel tetravalent bispecific antibodies. Nat Biotechnol 1997; 15:179-83; PMID:9305114; http://dx.doi.org/10.1038/nbt0997-159.

31. Orcutt KD, Ackerman ME, Cieslewicz M, Quiroz E, Sliusarczyk AL, Frangioni JV, et al. A modular IgG-scFv bispecific antibody topology. Protein Eng Des Sel 2010; 23:221-8; PMID:20190288; http://dx.doi.org/10.1093/protein/gp077.

32. Davis JH, Apericio C, Li Y, Kurusawa E, Lan Y, Lo KM, et al. SEED/bodies: fusion proteins based on strand-exchange engineered domain (SEED) CH, heterodimers in an Fn analogue platform for asymmetric binders or immunofusions and bispecific antibodies. Protein Eng Des Sel 2010; 23:195-202; PMID:20299542; http://dx.doi.org/10.1093/protein/gp094.

33. Moore GL, Baustica C, Pong E, Nguyen DHT, Jacinto J, Ezrati A, et al. A novel bispecific antibody format enables simultaneous bivalent and monovalent co-engagement of distinct target antigens. Mabs 2013; 5; in press; PMID:22212955.

34. Kontermann RE. Alternative antibody formats. Curr Opin Mol Ther 2010; 12:1716-8; PMID:20373621.

35. Kellner C, Bruenke J, Stieglmayer J, Schwemle M, Schwenk M, Singer H, et al. A novel CD19-directed recombinant bispecific antibody derivative with enhanced immune effector functions for human leukemic cells. J Immunother 2008; 31:871-84; PMID:18583300; http://dx.doi.org/10.1097/JIT.0b013e31818679d9.

36. Holliger P, Prospero T, Winter G. ‘Diabodies’, small bivalent and bispecific antibody fragments. Proc Natl Acad Sci USA 1999; 96:6444-8; PMID:8341653; http://dx.doi.org/10.1073/pnas.96.14.6444.

37. FritzKerald G, Holliger P. Improved tumour targeting by disulphide-stabilized diabodies expressed in Pichia pastoris. Protein Eng 1997; 10:1221-5; PMID:9488147; http://dx.doi.org/10.1093/protein/10.1093/protein.10.1221.

38. Johnson S, Burke S, Huang L, Gorlats L, Li H, Wang W, et al. Effector cell recruitment with novel Fv-based dual-affinity re-targeting protein leads to potent tumor cytolyis and in vivo B-cell depletion. J Mol Biol 2010; 399:436-49; PMID:20382161; http://dx.doi.org/10.1016/j.jmb.2010.04.001.

39. Brusselbach S, Korn T, Volkel T, Muller R, Kontermann RE. Enzyme recruitment and tumor cell killing in vitro by a secreted bispecific single-chain diabody. Tumor Targeting 1999; 4:115-23.

40. Kipriyanov SM, Moldenhauer G, Schuhmacher J, Cochlovius B, Von der Lieth CW, Marx ER, et al. Bispecific tandem diabody for tumor therapy with improved antigen binding and pharmacokinetics. J Mol Biol 1999; 293:41-56; PMID:10512714; http://dx.doi.org/10.1006/jmbi.1999.3156.

41. Schoonjans R, Willems A, Schoonooghe S, Leen J, Groosen J, Mertens N. A new model for intermediate membrane-spanning domains of bispecific and trispecific antibodies by efficient heterodimerization of single chain variable domains through fusion to a Fab-chain. Biomol Eng 2001; 17:193-202; PMID:11337278; http://dx.doi.org/10.1016/S0940-0018(01)00666-1.

42. Goldenberg DM, Rossi EA, Sharkey BM, McBride WJ, Chang CH. Multifunctional antibodies by the Dock-and-Lock method for improved cancer imaging and therapy by pertargeting. J Nucl Med 2008; 49:158-63; PMID:18077530; http://dx.doi.org/10.2967/jnumed.107.046185.

43. Kontermann RE. Strategies to extend plasma half-lives of recombinant antibodies. BioDrugs 2009; 23:93-109; PMID:19489651; http://dx.doi.org/10.1007/s40265-010-0003-0.

44. Kontermann RE. Strategies for extended serum half-life of protein therapeutics. Curr Opin Clin Pharmacol 2007; 1:590-602; PMID:17898890; http://dx.doi.org/10.1038/ncopharmacol.2007.43.
70. [Citation Text]
71. [Citation Text]
72. [Citation Text]
73. [Citation Text]
74. [Citation Text]
75. [Citation Text]
76. [Citation Text]
77. [Citation Text]
78. [Citation Text]
79. [Citation Text]
80. [Citation Text]
81. [Citation Text]
82. [Citation Text]
83. [Citation Text]
84. [Citation Text]
85. [Citation Text]
86. [Citation Text]
87. [Citation Text]
88. [Citation Text]
89. [Citation Text]
90. [Citation Text]
91. [Citation Text]
92. [Citation Text]
93. [Citation Text]
94. [Citation Text]
95. [Citation Text]
96. [Citation Text]
97. [Citation Text]
98. [Citation Text]
99. [Citation Text]
100. [Citation Text]
101. [Citation Text]
102. [Citation Text]
103. [Citation Text]
104. [Citation Text]
105. [Citation Text]
106. [Citation Text]
107. [Citation Text]
108. [Citation Text]
109. [Citation Text]
110. [Citation Text]
111. [Citation Text]
112. [Citation Text]
113. [Citation Text]
114. [Citation Text]
115. [Citation Text]
116. [Citation Text]
117. [Citation Text]
118. [Citation Text]
119. [Citation Text]
120. [Citation Text]
121. [Citation Text]
122. [Citation Text]
123. [Citation Text]
124. [Citation Text]
125. [Citation Text]
126. [Citation Text]
127. [Citation Text]
128. [Citation Text]
129. [Citation Text]
130. [Citation Text]
131. [Citation Text]
132. [Citation Text]
133. [Citation Text]
134. [Citation Text]
135. [Citation Text]
136. [Citation Text]
137. [Citation Text]
138. [Citation Text]
139. [Citation Text]
140. [Citation Text]
141. [Citation Text]
142. [Citation Text]
143. [Citation Text]
144. [Citation Text]
145. [Citation Text]
146. [Citation Text]
147. [Citation Text]
148. [Citation Text]
149. [Citation Text]
150. [Citation Text]
151. [Citation Text]
152. [Citation Text]
153. [Citation Text]
154. [Citation Text]
155. [Citation Text]
156. [Citation Text]
157. [Citation Text]
158. [Citation Text]
159. [Citation Text]
160. [Citation Text]
161. [Citation Text]
162. [Citation Text]
163. [Citation Text]
164. [Citation Text]
165. [Citation Text]
166. [Citation Text]
167. [Citation Text]
168. [Citation Text]
169. [Citation Text]
170. [Citation Text]
171. [Citation Text]
172. [Citation Text]
173. [Citation Text]
174. [Citation Text]
175. [Citation Text]
176. [Citation Text]
177. [Citation Text]
178. [Citation Text]
179. [Citation Text]
180. [Citation Text]
181. [Citation Text]
182. [Citation Text]
183. [Citation Text]
184. [Citation Text]
185. [Citation Text]
186. [Citation Text]
187. [Citation Text]
188. [Citation Text]
189. [Citation Text]
190. [Citation Text]
191. [Citation Text]
192. [Citation Text]
193. [Citation Text]
194. [Citation Text]
195. [Citation Text]
Biotechnol Prog 2005; 21:205-20; PMID:15903260; http://dx.doi.org/10.1023/A:1025009300562.

Doughty BL, Kapo SW, Ranganathan S, Reece DA, Joned Y, Wang TH, et al. Preclinical manufacture of a bispecific antibody-toxin conjugate targeting human aminopeptidase N. Cancer Res 2007; 67:5482-9; PMID:17775161; http://dx.doi.org/10.1158/0008-5472.CAN-06-2454.

Stish BJ, Chen H, Shu Y, Ohlsfeldt JR, Vallera DA. A novel bispecific antibody-toxin conjugate targeting human aminopeptidase N. Cancer Res 2007; 67:5482-9; PMID:17775161; http://dx.doi.org/10.1158/0008-5472.CAN-06-2454.

Dougan MJ, Dronoff G. Immune therapy for cancer. Annu Rev Immunol 2009; 27:83-117; PMID:19007331; http://dx.doi.org/10.1146/annurev.animmunol.2009.030911.145608.

Oritz-Sánchez E, Helguera G, Daniels TR, Penichet ML. Antibody-cytokine fusion proteins: applications in cancer therapy. Expert Opin Biol Ther 2008; 8:669-32; PMID:18407765; http://dx.doi.org/10.1517/14712598.8.5.609.

Radchenko, FK, Tabilin, MV, Igumnov, AV, Tverskaya, O, Schegoleva, T, Postnova, VA, Kirpotin, DP, Karpukhin, AV, Scherphof, GL, et al. Interaction of differentially designed immunotoxins with colon cancer cells and Kupffer cells. An in vitro comparison. Pharmacol Res 2003; 48:383-9; PMID:12856452; http://dx.doi.org/10.1016/S0031-6975(02)00073-5.

Gillies RC, zum Gath, AM, Bogh, J, et al. Preclinical evaluation of a bispecific antibody-toxin conjugate targeting human aminopeptidase N. Cancer Res 2007; 67:5482-9; PMID:17775161; http://dx.doi.org/10.1158/0008-5472.CAN-06-2454.

Dougan MJ, Dronoff G. Immune therapy for cancer. Annu Rev Immunol 2009; 27:83-117; PMID:19007331; http://dx.doi.org/10.1146/annurev.animmunol.2009.030911.145608.

Oritz-Sánchez E, Helguera G, Daniels TR, Penichet ML. Antibody-cytokine fusion proteins: applications in cancer therapy. Expert Opin Biol Ther 2008; 8:669-32; PMID:18407765; http://dx.doi.org/10.1517/14712598.8.5.609.

Radchenko, FK, Tabilin, MV, Igumnov, AV, Tverskaya, O, Schegoleva, T, Postnova, VA, Kirpotin, DP, Karpukhin, AV, Scherphof, GL, et al. Interaction of differentially designed immunotoxins with colon cancer cells and Kupffer cells. An in vitro comparison. Pharmacol Res 2003; 48:383-9; PMID:12856452; http://dx.doi.org/10.1016/S0031-6975(02)00073-5.

Gillies RC, zum Gath, AM, Bogh, J, et al. Preclinical evaluation of a bispecific antibody-toxin conjugate targeting human aminopeptidase N. Cancer Res 2007; 67:5482-9; PMID:17775161; http://dx.doi.org/10.1158/0008-5472.CAN-06-2454.

Dougan MJ, Dronoff G. Immune therapy for cancer. Annu Rev Immunol 2009; 27:83-117; PMID:19007331; http://dx.doi.org/10.1146/annurev.animmunol.2009.030911.145608.
146. Saul JM, Annapragada AV, Bellamkonda RV. A dual-ligand approach for enhancing targeting selectivity of therapeutic nanocarriers. J Control Release 2006; 114:277-87; PMID:16904220; http://dx.doi.org/10.1016/j.jconrel.2006.05.028.

147. Quan C, Chang C, Wei H, Chen CS, Xu XD, Cheng SX, et al. Dual targeting of a thermosensitive nano-gel conjugated with transferrin and RGD-containing peptide for effective cell uptake and drug release. Nanotechnology 2009; 20:335101; PMID:19636104; http://dx.doi.org/10.1088/0957-4484/20/33/335101.

148. Meng S, Su B, Li W, Ding Y, Tang L, Zhou W, et al. Enhanced antitumor effect of novel dual-targeted paclitaxel liposomes. Nanotechnology 2010; 21:415103; PMID:20852556; http://dx.doi.org/10.1088/0957-4484/21/41/415103.

149. Klaza E, van der Schaft DWJ, Hautvast PAJ, Mulder WJM, Mayo KH, Griffioen AW, et al. Synergistic targeting of alphavbeta3 integrin and galectin-1 with heteromultivalent paramagnetic liposomes for combined MR imaging and treatment of angiogenesis. Nano Lett. 2010; 10:52-8; PMID:19968235; http://dx.doi.org/10.1021/nl902659g.

150. Murase Y, Asai T, Katanasaka Y, Sugiyama T, Shimizu K, Maeda N, et al. A novel DDS strategy, “dual-targeting”, and its application for antineovascular therapy. Cancer Lett 2010; 287:165-71; PMID:19616372; http://dx.doi.org/10.1016/j.canlet.2009.06.008.

151. Friedman M, Lindström S, Ekerljung L, Andersson-Svahn H, Carlsson J, Brismar H, et al. Engineering and characterization of a bispecific HER2 x EGFR-binding affibody molecule. Biotechnol Appl Biochem 2009; 54:121-31; PMID:19492986; http://dx.doi.org/10.1042/BA20090096.