International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Review Article

Bispecific Antibodies: Progress and Application

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ABSTRACT

Bispecific antibodies also termed as "dual targeting" or "dual specificity" antibodies are one of the fastest growing classes of investigational drugs with around 100 different formats in the development. However, only two of these, Blinatumomab and Catumaxomab are approved till date. Most of the bispecific antibodies are being investigated for oncological indication with around 20 being investigated for non-oncological indications. BsAbs show therapeutic effect by various mechanisms of action such as blocking two different antigens or mediators that play an important role in disease pathogenesis, inducing cell signalling pathways such as in proliferation or inflammation, retargeting to mediate Antibody dependent cell-mediated cytotoxicity, by delivering toxicities or by engaging T-cells. And some of the bispecific antibodies are designed in a way so as to improve the pharmacokinetic properties (PK-modulating BsAbs). In this review, we focus on the mechanism of action of bispecific antibodies that are approved or are in clinical trials and discuss the progress and latest advances in the development of these drugs.

Key words: Bispecific antibodies, BsAbs.

INTRODUCTION

Bispecific antibodies possess the capability of binding simultaneously two different antigens or two distinct epitopes on a same antigen. In case of same antigen the effector site may bind to molecules such as antigens, enzymes, drugs, cytokines, toxins, radio nucleotides, plasma proteins and cells such as t cells, NK cells, macrophages and neutrophils. Whereas the targeting site may bind to molecules such as cytosine, growth factors, cellular targets like receptors and adhesion molecules and infectious agents. [1] By simultaneously recognizing two different targets, bsAbs can act as mediators to redirect immune effector cells, such as Natural Killers cells and T-cells, to tumor cells, enhancing their destruction. In addition, by targeting two different receptors in combination on the same cell, bsAbs can cause modifications of cell signaling, including the inactivation of proliferation or inflammatory pathways. [2] BsAbs do not usually occurs in nature and are required to be created through the use of recombinant technology or by somatically fusing two hybridomas (hybrid hybridoma / quadroma) or through chemical means. [3]

Based on the presence or absence of Fc domain, bispecific antibodies are divided into two types, IgG-like and non IgG-like bispecific antibodies. IgG-like bsAbs have conserved Ig constant domain, thus these bear an Fc region which helps in purification of the bsAb using protocols established for IgG molecules, and can contribute to improved solubility and stability. Also these antibodies can exhibit Fc mediated effector functions, which might be desirable add-ons for therapeutic applications, such as antibody-dependent cellular cytotoxicity (ADCC), complement fixation (CDC), and enhanced long half-life resulting from their larger size and FcRn-

mediated recycling processes. Small bispecific antibodies that lack constant domain on the other hand rely entirely on their antigen-binding capacity to exert their therapeutic activities. These bsAbs have been primarily designed as effector cells recruiters and T-cells engagers. [4]

BsAbs were first developed by Quadroma or Hybrid hydromas approach in 1980s. This technology consisted of producing bsAbs by somatic fusion of an antibody-drug producing lymphocyte and a myeloma cell. Later a chimeric quadroma was created from a murine and a rat hybridoma cell line with some improvements. Recently a more advanced method has been described using mild reducing agent 2-mercaptoethanesulfonic acid sodium salt to produce bsAbs from mAbs of a different or the same subclass and same species rapidly. In addition to having two antigen-binding sites for different antigens the intact Fc region can bind to monocytes/ macrophages, natural killer cells, dendritic cells or other Fc receptor expressing cells. Thus, because there are three regions binding, this type of bsAbs is called trifunctional bispecific antibody or triomabs. [5,6]

Another engineering is knobs-into-holes which is based on transfection of modified human antibody coding genes in mammalian cells. Advantage over Quadroma approach is that there is less immunogenicity as the formed bsAbs are of human nature instead of murine/ rat nature. The technology consists of developing heavy chain homodimers for heterodimerization. It consists of creating 'knob' variant by replacing small amino acid (T3664) on the CH3 domain of CD4-IgG immunoadhesin with large amino acid (Y407). 'Hole' is produced by replacing large amino acids with smaller amino acid in the CH3 domain of humanised anti-CD3 antibody. And then, the knob is inserted into the hole.To optimise heavy chain association and stability of bsAbs some mutations has been developed in the past, two in knob heavy chain and four in hole heavy chain. Also the heterodimeric Fc part can be stabilised by adding artificially created disulfide bridges. [5,6]

To ensure the light chain association apart from ensuring heavy chain association a modification of the above method was introduced called CrossmAb. In this technique the heavy and light chains of only one arm are modified by immunoglobulin domain crossover (Crossover of the antibody domain within one Fab arm of a bispecific IgG antibody whereas the heavy chain association can be corrected by Knobs-into-holes, electrostatic steering and other alternative approaches. This approach allows generation of various formats of bispecific antibodies including bi (1+1), tri (2+1) and tetra (2+2) antibodies and also non Fc tandem Fab based antibodies. Various alternative technologies have been developed further after the description of CrossmAb technology allowing generation of IgG based bispecific antibodies from any pair of antibodies. Some of these approaches include DVD-IgG and CODV-IgG, duo bodies, Dual action FA's (DABs) or Dutadabs etc. Numerous CrossmAbs have been evaluated in preclinical studies, advanced four of which are currently in active phase 1/2 clinical trials. [6,7]

Dual-variable-domain immunoglobulin (DVD-Ig) format was first described in 2007. In this method the target binding variable domains of two preexisting monoclonal antibodies are combined through naturally occurring linkers to create a tetravalent antibody having dual specificities. The DVD-IG bsAbs preserve the affinities of parent monoclonal antibodies, hence both the antigen binding sites can function independent of one another as there is no stearic hindrance posed. Monoclonal antibodies of different nature (Human, murine or Chimeric) can be combined/used in generating DVD-Ig bispecific antibodies. [6,8]

Bispecific T-Cell engager antibodies (BiTEs) consists of fusing only the variable regions of monoclonal antibodies in the former of Single chain Fvs (ScFvs) by

linker peptides. DARTs are diabodies like molecules. In these the VH of the first variable region is linked to the VH of second binder and the VH of second variable regionis linked to VL of the first binder. Stabilization is achieved by introducing additional disulfide bridges. TandAbsarebi specific fusion proteins with four binding sites. Two of the binding sites are for binding to tumor cells surface antigens and the other two bind to immune cells. [6]

MECHANISM OF ACTION OF BISPECIFIC ANTIBODIES:

1. Activating and Redirecting Cellular Immunity towards the Tumor Cells:

The Bispecific antibodies showing this function activates and recruits the unstimulated effector cells of immune system for destruction of tumor cells bearing the target antigens. One of the antigen binding sites identifies and binds to the target antigen on the tumor cells and the other site binds to the suitable leukocyte. T-Cells expressing CD3 and CD4 and NK Cells expressing mostly CD16 are frequently recruited leukocytes by bispecific antibodies. [9]

Bispecific T-Cell engagers (BiTEs) are found to be very efficient in recruiting the T-Cells and binding to tumor associated antigen enhancing the killing of the malignant cells. One scFv binds to the T-Cell specific molecule, usually CD3 while the other scFv binds to a tumor associated antigen. Initial recognition events include binding of T-cell to CD3 binding fragment and tumor associated antigen. This activates polyclonal T-cells in a non-MHC-restricted fashion in the presence of appropriate costimulatory receptors such as CD28,OX-40,4-1BB etc to their cognate ligands viz. B7-1,B7-2,OX-40,4-1BBL etc. Other antibodies that can facilitate the activation of T-cells are anti-CD-28, anti-4-1BB etc by triggering the costimulatory receptors. Tcell activation by the signals is followed by their degranulation releasing perforins, granzymes and lysosomal enzymes which lead to apoptosis of the target cells. [9,10]

In addition to costimulatory receptor T-cells also express inhibitory receptors (CTLA4, PD1etc) that on binding to their cognate ligands (B7-1,B7-2,PD-L1 etc)on target cells can inhibit the activation of T-cells. This can be prevented by non activating antibodies which can bind to either the co-inhibitory receptors (anti-CLA4, anti-PD1) or to their ligands (anti-PD-L1, Anti-B7-1, anti-B7-2 etc). Effector memory cells are the most commonly involved T-cells in the tumor killing. They work by same mechanism except that they're already activated and just require stimulation via CD3 signalling. [11]

Blinatumomab is one such BiTE that, for its excellent effects in clinical trials achieved an accelerated approval from the FDA in December 2014 for the treatment of children and adults with Philadelphia chromosome-negative relapsed (that had returned after earlier treatment) or refractory (those that failed to respond to standard therapies at all) B-cell precursor acute lymphoblastic leukemia. However on July 12, 2017under the change to full approval, FDA expanded the indication for blinatumomab to patients with Philadelphia chromosome-positive ALL. One scFv binds to CD19 positive lymphocytes, while the other binds to unstimulated primary human CD3. Aphase 3 randomised, open label multicenter clinical trial (TOWER, NCT02013167) was conducted in 405 patients with relapsed or refractory B-cell ALL. Blinatumomab was administered at 9mcg/day by continuous intravenous infusion for the first 7 dats of study followed by an escalated dose of 28mcg/day starting from week 2 of treatment. Statistically significant improvement was observed in patients treated with blinatumomab compared to those treated with Standard of care chemotherapy. The long-term follow-up part of the study was discontinued based on a recommendation from an independent data monitoring committee (DMC) that the study be stopped

for benefit. It was also found to be effective for patients who have recurred after chemotherapy and after allogeneic hemopoietic stem cell transplantation. Patients having non-Hodgkin's lymphoma also showed tumor regression with blinatumomab. [12]

MT111/AMG 2111/ MEDI-656 targets CEA, an immunoglobulin superfamily glycoprotein that is expressed on a variety of solid tumors and on GI tumors.CEA (Carcinoembryonic antigen) is a glycosylated lumen oncofetal antigen (antigen in normal cells is present in low concentrations and only in luminal portions of cell) belonging to CEA related cell adhesion molecules(CEACAM) family of IgG gene superfamily. MT111 binds CEA and CD3. In a multicenter phase 1, open label study, 39 patients with advanced GI adenocarcinoma were given MEDI 656 intravenously over 3 hours on days 1 through 5 in 28 day cycles with dexamethasone premedication. At the end of the study 11 patients had stable disease. The median overall survival for 39 patients was 5.5 months and MTD of MEDI656 was 5mg. Nausea, Vomiting, abdominal discomfort and fatigue were considered most common adverse effects. High level antidrug antibodies were found in 19 patients. It showed rapid clearance and a short half life. [13]

Solitomab (MT110/AMG110), BAY2010112(AMG212/MT112), MOR209/ES414 are the other BiTEs which entered into clinical trials. Solitomab completed phase 1 while MT112 and MOR209 are in the phase 1 of clinical trials. Solitomab can bindto polyclonal CD8+ and CD4+ T-cells for highly potent redirected lysis of target cells. The mechanism involves membrane damage, membrane blessing, activation of procaspases 3 and 7, fragmentation of nuclear DNA and cleavage of caspase substrate poly (ADP ribose) polymerase. Preclinical studies with MT110 with a mouse surrogate molecule (muS110) have confirmed the efficacy of these molecules in the inhibition of metastases formation. [14]

Dual affinity re-targeting (DART) molecules are another set of bispecific antibodies which acts by recruiting immune effector cells. The size of DART molecules is larger than that of BiTEs and this contributes to serum stability and extended storage. MGD006 is a CD123×CD3 bispecific antibody currently in phase 1 of the clinical trials for relapsed/refractory Acute Myeloid Leukemia (AML) or intermediate-2/High risk myelodysplastic syndromes. Other bsAbs of DART format which are currently in the phase 1 of clinical trials are MGD007 which is a GPA33×CD3 binding, PF-06671008 which is p-cadherin×CD3 binding, MGD009 that is B7H3×CD3 engaging, the former being studied against colorectal cancer and the latter two against solid tumors. Duvortuxizumab (MGD011, JNJ64052781) is CD19×CD3 binding DART currently in the part-2 (dose expansion) phase-1 of clinical trials for relapsed/refractory B-cell malignancies. [15]

TandAbsare tetravalent bispecific CD19/CD3 tandem diabodies comprising solely of Fvdomains, having two binding sites for each antigen. AFM13 is a NK-Cell recruiting TandAb, Fc portion of which binds to CD30 on the tumor cells and Fcγ receptor IIIA (CD16A) and trigger antibody dependent cellular cytotoxicity (ADCC). CD16A is an antigen expressed on NK-Cell and macrophages and not on neutrophils. The crosslinking of CD30+ tumor cells and NK-cells causes the activation of cytolytic activity of NK-cells. The cytolytic granules such as perforins and granzymes are released. Perforins causes the formation of pores in the target cells, cell lysing components enter the cell and cause destruction. [16,17] AFM13 is now being studied in the phase 2 clinical trials as a monotherapy against relapsed or refractory Non-Hodgkin's lymphoma and in phase 1 in combination with Merck's keytruda. AFM11 is a T-Cell binding TandAb with two binding sites for CD3 and two for

CD19. By mechanism similar to AFM13 it forms an immune synapse between CD3 and CD19 and causes destruction of tumor cells expressing CD19. AFM11 entered phase 1 of clinical trial for Relapsed or refractory CD19 positive B-cell Non-Hodgkin's lymphoma currently recruiting participants. (Clinicaltrials.gov NCT02665650, NCT02321592NCT02106091).

Triomabs are another set of bispecific antibodies that enables immune redirection towards tumors by the formation of a tricellular complex comprising T-cells, tumor cells and Fc receptor expressing accessory cells. Catumaxomabis a non humanised (rat/murine hybrid), trifunctional, bispecific monoclonal antibody combining two half antibodies of both mouse and rat origin approved by European union in April, 2009 for the intraperitoneal treatment of malignant ascites in patients with EpCAM positive epithelial tumors for which a standard therapy is not available or is no longer feasible. One Fab fragment (part of mouse hybridoma expressing anti-tumor EpCAM antibody, IgG2a) binds to EpCAM expressed on most epithelial tumors of non squamous differentiation and the other fragment (part of rat hybridoma cell line producing anti-tumor CD3 antibody, IgG2b) binds to CD3 of T-cell receptor complex. The Fc region binds to accessory cells expressing Fc gamma receptor I and III like macrophages, dendritic cells and NK Cells. Formation of an immunological tricellular complex leads to release of cytokines derived from immune cells including IL-1 β, IL-2, IL-6, IL-12 and dendritic cell cytokine-1.The side effects were manageable, generally short lived and rarely severeand most were related to cytokine rekease such as fever, chills, nausea, vomiting, fatigue and abdominal pain. [18] Catumaxomab was found to be safe and appropriate in treating both hospitalised and out patients. [19]

cLc-Hetero-H chain IgG bispecific antibodies MCLA117 (anti-CLEC12A×anti-CD3) for acute myelogenous leukemia, REGN1979(anti-CD20×anti-CD3)for B-cell malignancies and ERY974 (anti-GPC3×anti-CD3) for advanced solid tumors are in the clinical trials phase 1 currently recruiting participants. [15] (NCT03038230, NCT02290951, NCT02748837)

scFv-Fc-Fab fused bispecific antibodies such as Xmab 14045 (anti-CD123×anti-CD3) for CD123 expressing haematological malignancies and GBR1392 (anti-Her2×anti-CD3) for HER2 positive carcinomas are in phase 1 of clinical trials currently recruiting participants. [15] (NCT02730312, NCT02829372).

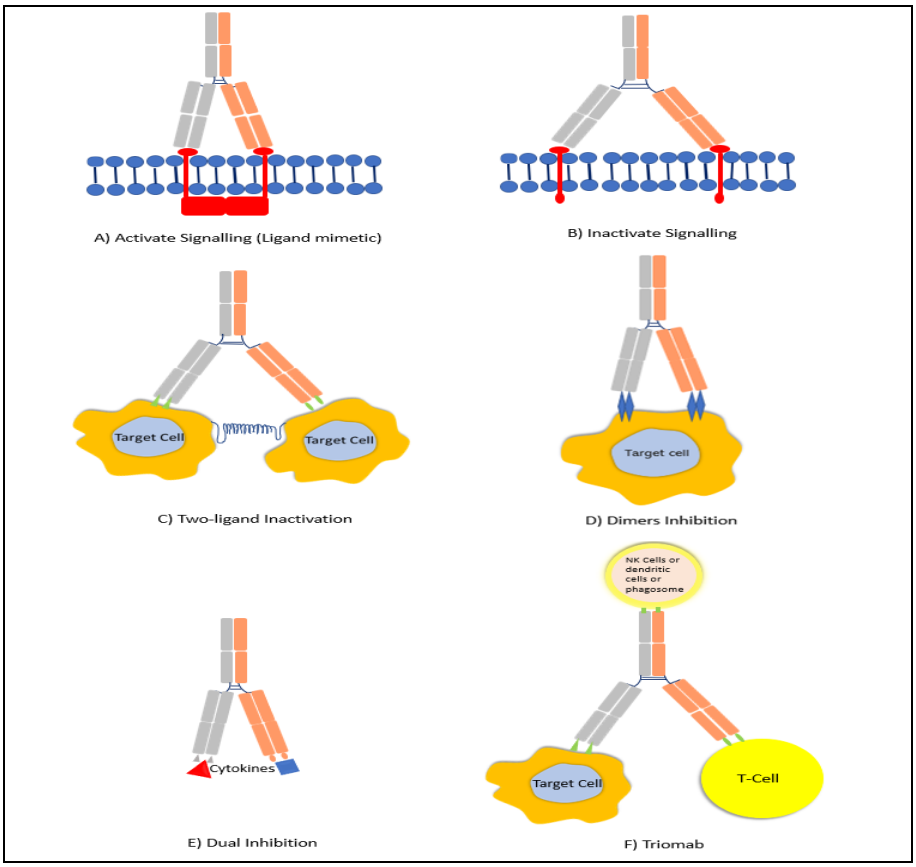
BTCT44654 being studied for Non-Hodgkin's lymphoma(NHL) and chronic lymphocytic lymphoma (CLL) as a single agent and in combination with Atezolizumab (NCT02500407). JNJ 63709178 is being studied in phase 1 Clinical trial currently recruiting participants.

2. Delivering Cytotoxic Substances to the Malignant Cells:

BsAbs that bind to the cell surface antigens and digoxigenin simultaneously are generated for targeted or pretargeted payload delivery. In this approach the targeting molecules are IgGs that bind to tumor antigens including Her2, IGFIR, CD22 or LeY. Payloads are digoxigeninylated which comprises small compounds (Dig-Cy5, Dig-Doxorubicin) and proteins (Dig-GFP). Bispecific antibodies carrying unmodified hapten binding module thats form non covalent complexes between delivery vehicles and payload and can be separated on internalisation into the cell. This intracellular payload release, facilitates the uptake and improve the activity of compounds whose molecular targets are located intracellularly. The additional stabilization of payloads by disulfide bonds renders stability in the circulation and minimizes undesired premature payload release. Once the complex reaches inside cells the payloads can be released by reduction in disulfide bonds. Hapten based

bispecific antibodies deliver siRNA (Short-interfering RNA) effectively into the tumor cells which knocks down some vital genes and can be considered a promising approach for cancer therapy. SiRNA is haptenylated by digoxigenin at its 3' end and formulated into nanoparticles consisting of dynamic polyconjugates (DPCs) or into Lipid-based nanoparticles (LNPs). This is then completed with bsAbs in defined ratio of 2:1. The complex specifically targets cells expressing corresponding antigen and is then internalised into endosomes where the Dig-siRNAs are separated from bsAbs. [20] Tung et al. generated polyethylene glycol (PEG) binding bsAbs. PEG is a polymer that can be linked to various payloads such as peptides, proteins, liposomes and nanoparticles in same manner as standard haptens and hence can be used for targeted nanoparticles delivery. In this the nanoparticles are coupled to other haptens. [21]

Another method of payload delivery through hapten binding is pretargeting in which the targeting vehicles are administered first which gets distributed and bound to target sites and the non bound targeting vehicles are cleared from circulation. This is followed by administration of haptenylated payloads which becomes captured at the desired target sites by hapten binding bsAbs. Initially pretargeting was done based on conjugation of avidin/streptavidin modules to bsAbs for accumulating biotinylated (radioactive) payloads on target tissues. But this method induced immunogenicity due to nonhuman haptens which was later improved by using standard haptensbinders. HSG(Histamine-Succinyl-Glysine) haptens conjugated with chelating moieties such as DTPA and DOTA were radiolabelled with In-111 and Ln-177/Y-90 respectively for pretargeted radioimmunotherapy. A study validated a SPECT/CT- based therapeutic PRIT (pretargeting radioimmunotherapy) regimen -177Lu-DOTA-Bn that led to 100% complete response and cure without any treatment related toxicities with high therapeutic indices for radiosensitive tissues such as blood and kidney. [20,22]



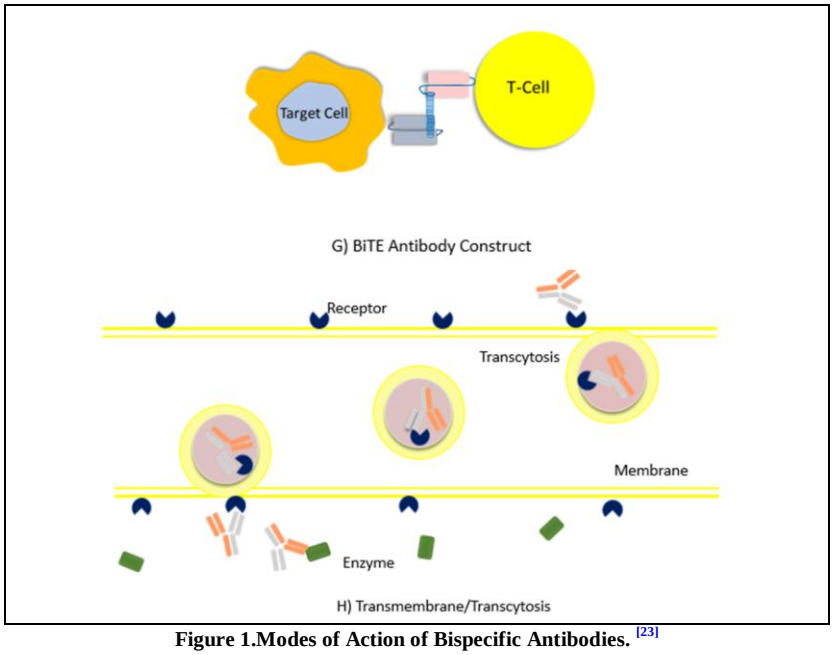


Figure 1.Modes of Action of Bispecific Antibodies. [23]

A) Activate signalling by a ligand mimetic; B) Inactivate signalling of two ligands or receptors by binding to them; C) Two-ligand inactivation: Two arms of the bispecific antibody binds to different ligands on different cells belonging to the same population, such as DLL4 ×VEGF, TNFα × IL17A, IL14 × IL13 etc; D) Dimers Inhibition: BsAbs can bind to two receptor/ligands on the same cell (HER2×HER3, HER2,HER4) eg. MM-111; E) Dual Inhibition: BsAbs can inhibit two different cytokines simultaneously, for example, COVA322 that inhibits TNFα and IL17A; F) The antigen binding sites of BsAb binds to Target cell receptor and T-Cell receptor; Heavy chain site binds to NK cells or dendritic cells or macrophages/phagosomes (e.g., Catumaxomab, ertumaxomab, FBTA05); G) BiTEs bridges Target cells and T cells by binding to CD3/CD28 or CD19/CD20/CD22/CEA/EPCAM, respectively (e.g. Blinatumomab, MEDI-565, MT110); H) Transmembrane/Transcytosis: BsAbs cross the membrane via receptor transport (Transferrin receptor TfR) and bind to enzyme receptor on the other side (BACE1).

DT-2219 is a bispecific recombinant immunotoxin containing the catalytic and translocation enhancing domain of diphteria toxin (DT390) fused with bispecific Single chain Fv of antibodies targeting human CD19 and CD22. It internalize into cell readily helping toxin entry into the cytosol causing inhibition of protein synthesis and subsequent cell death through apoptosis. Unlike cytostatic chemotherapies DT2219-mediated tumor toxicity is independent of cell cycle and p53 signalling. [24]

Minicells are 400±20nm sized bacterially derived anucleated nanoparticles created by inactivation of genes that controls normal cell division in bacteria. These can be packaged with therapeutically significant concentrations of chemotherapeutics, siRNA or shRNA after complete and reproducible purification through procedures used to eliminate contaminants such as parenteral bacterial cells, debris etc, cross flow filtration and centrifugation. These minicells loaded with chemotherapies is targeted to tumor cells through attachment of bsAbs to minicells surface. One arm of bsAbs has specificity towards liposaccharide content of minicells and the inherent arm has specialty towards the tumor cells surface receptor. The minicell bound to bsAbs is then internalised, enfocytysed and degraded in the lysosomes. The minicell releases the cytotoxic drug allowing the tumor cell to commit suicide. Preclinical studies in xenograft models

showed significant tumor killing by EGFR-targeted-Paclitaxel-loaded minicells. Also open label, phase 1 study in patients with advanced solid tumors was conducted in which it was found to be safe and showed clinical efficacy. [25,26]

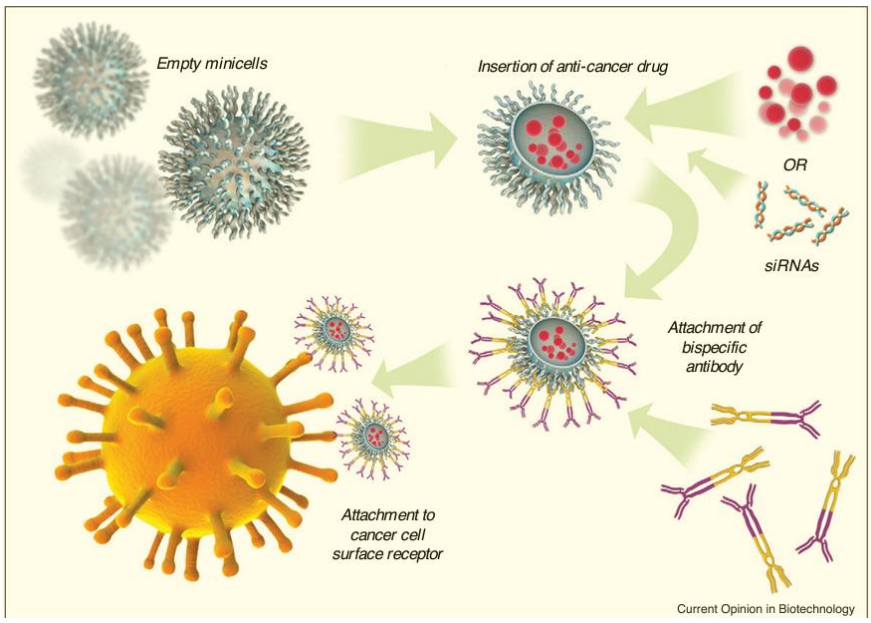


Figure 2: Bispecific Antibody targeted, drug/SiRNA-packaged Minicells. [29]

Schematic showing the packaging of anti-cancer drugs or SiRNAs into empty minicells and targeting them to tumor cell-surface receptor using bispecific antibodies where one arm of the antibody has minicell-surface O-polychharide specificity and the other arm has specificity for the tumor cell-surface receptor for example EGFR.

Andreev J et al demonstrated that non covalently linking HER2 and PRLR at plasma membrane using anti-HER2×anti-PRLR bsAbs triggers HER2 degradation in the lysosomes and coupling an ADC to this rapidly internalising protein may be useful approach to enhance internalisation and cell killing activity. [27]

3. Overcoming Drug Resistance:

The resistance developed against anticancer drugs is likely due to inhibitory checkpoint molecules as well as crosstalk suggests between the signalling pathways. Many therapeutic strategies are being developed to overcome this resistance out of which bispecific antibodies are best choice. Studies revealed that herugulin (a HER3 ligand) induces resistance to GDC-0941, a PI3K targeting drug against prostate cancer. Bispecific antibody targeting HER2/HER3 is useful in overcoming this resistance and can restore the sensitivity of the drug GDC-0941 towards prostate cancer cells. [28,29]

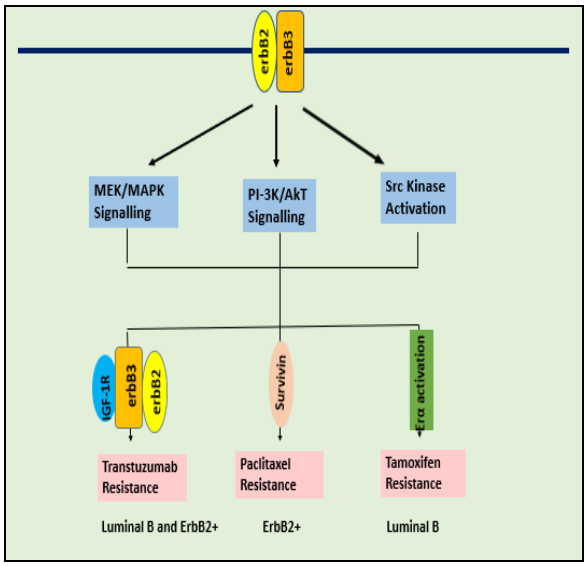


Figure 3: ErbB3(Her3) interacts with ErbB2(Her2) to activate signaling pathways leading to multi-drug resistance in breast cancer. [30]

In both luminal B and erbB2 subtypes of human breast cancer, erbB2/erbB3 association may recruit IGF-1R to form a trimeric complex activating PI-3K/AkT signalling and Src kinase and

resulting in transtuzumab resistance. (Huang: Cancer Res 2010); In erbB2+ breast cancer, association of erbB2 and erbB3 upregulates Survivin via a PI-3/ AkT-dependent mechanism, and thereby confers paclitaxel resistance. (Wang: Oncogene 2010); In luminal B breast cancer, the erbB2/erbB3 heterodimers modulate ERα phosphorylation and activation through MEK/MAPK and or PI-3 K/Akt signalling pathways and subsequently alter tamosifen sensitivity. (Liu: Int J Cancer 2007)

B7-H3 is a B7 family homologue 3, a transmembrane protein with IgG like structure belonging to B7 superfamily can activate or inhibition T-Cell responses. It is overexposed in malignant cells. Some studies revealed that B7-H3 exhibited inhibitory actions on host T-cells in cancer patients. BsAb targeting CD3 and B7-H3 was used to direct activated T-cells to kill tumor targets. In SCID-Beige mice model, ATC armed with B7-H3 bispecific antibody showed increased cytotoxicity and cytokines production suppressing B7-H3 positive cancer growth. [31]

Her2 is responsible for oncogenesis and resistance to treatment of various solid tumors. Andrew et al developed a HER2/CD3 targeting bsAbby replacing the variable region of hu3F8-bsAb with transtuzumab. In four humanized models of breast and ovarian cancer cells line xenograft and Patient derived xenografts of human breast and gastric cancer, it showed anti proliferative effects of transtuzumabas well as novel insensitivity to PD-1/PD-L1 immune checkpoint inhibition. [32]

Met oncogene have been reported to be involved in acquired resistance of tumors towards EGFR inhibitors. [33] To solve this bispecific antibodies targeting MET/EGFR and MET/HER2 were developed that successfully induced efficient internalisation of EGFR or HER2 and their degradation restoring the sensitivity of EGFR inhibitors. [34]

4. Inhibiting Multiple Signaling Ligands/Pathways:

Receptor tyrosine kinases (RTKs) are a superfamily of cell surface receptors that are involved in mediating intracellular signalling by phosphorylating substrate proteins involved in cell proliferation, diffentiation, survival and migration. Thus, can stimulate and modulate the growth of tumors. The Human Epidermal growth factor Receptors (HER) is a family in the superfamily RTKs comprising of EGFR (also known as ErbB1/HER1), HER2, HER3 and HER4. Although monospecific antibodies target EGFR, HER2 or other receptors are already in therapeutic practice, Cancer cells are able to escape the blocking of one signalling pathway by taking up other signalling pathways. [28]

Bispecific antibodies mediating simultaneous blockade of two pathways are now in Clinical development which are able to reduce the possibility of tumor escape from such mechanisms. The phosphorylation of EGFR and HER3 activates the downstream RAS/MAPK and PI3K/AKT signalling pathways which promotes cell growth and proliferation. Inhibition of either of the pathways alone cannot completely inhibit the growth and proliferation of tumor cells. Sliwkowski et al demonstrated the use of Duligotuzumab (MEHD7945A), a bispecific antibody in vitro and in vivo, the results of which revealed that MEHD7945A potently inhibits the phosphorylation of EGFR and HER3 as well as it enhances gemcitabine-mediated cytotoxicity. [35] It is currently in phase 2 of Clinical trials.

JNJ-61186372, a bispecific antibody targeting EGFR and cMET enhanced the killing of EGFR mutant lung cancer cells (NCT02609776).Bispecific antibody targeting EGFR and VEGFR2 showed potent antitumor activity by inhibiting receptors and blocking PI3K/AKT and MAPK signalling pathways. [36] Negrinet al investigated the ability of bispecific antibody targeting HER2 and Cancer Antigen-125 (CA-125) with CIK cells against primary ovarian carcinomas. Results

showed that the cytotoxic activity of CIK cells with BsAbs was much higher than CIKalone. [37]

5. Inhibition of tumor angiogenesis:

Angiogenesis, the formation and development of new blood vessels is stimulated and regulated by the release of specific growth factors from the tumor cells, endothelial cells or associated macrophages. These growth factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, transforming growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, epidermal growth factor etc. Out of all these VEGF is considered most powerful angiogenic agent in neoplastic tissues, as well as in normal tissues, whose expression is also associated with wide range of tumors in human. Production of these growth factors is associated with upregulation of physiological conditions associated with tumors such as hypoxia resulting from the increasing distance between the growing tumor cells and the capillaries or from the inefficiency of new vessels. Binding of vascular endothelial growth factor A (VEGF-A) to its endothelial receptors leads to the progression of tumor, angiogenesis and vascular permeability. Angiopoietin 2 is primarily secreted by endothelial cells. It is known to destabilise vessel assembly, increase vascular permeability, and cause endothelial cells sprouting and proliferation. Its upregulation is evident in wide range of malignancies. In some preclinical and clinical studies VEGF inhibitors alone were shown to be promoting tumor invasion and metastasis by increasing the intratumoral hypoxia. Simultaneously blocking two or more angiogenetic factors can increase therapeutic efficacy.

Vanucizumab/RG7221 and RG7716 are bispecific antibodies targeting Ang-2/VEGF currently being studied in phase 2 of clinical trials in patients with Colorectal cancer and Wet Age-related macula degeneration (AMD) respectively. (Clinicaltrials.gov Id: NCT02141295, NCT03938880). It was also demonstrated that simultaneous inhibition of VEGF/Ang-2 normalises tumor vasculature and prolongs survival in patients with glioblastoma by altering macrophages and resident microglia. [38]

DLL4, Delta like ligand-4 is a notch ligand which also plays an important role in vascular development. DLL4/Notch signalling pathway regulates vasculature development as well as tumor angiogenesis. Apart from VEGF gene, DLL4 is the only gene whose haploinsufficiency leads to embryonic lethalities and vascular defects. However, there are many genes involved in vascular development. Downstream of the VEGF/VEGF signalling pathway DLL4/Notch pathway plays a key negative regulator. VEGF signalling pathway activates the Notch pathway by up regulating DLL4 expression. The Notch activation causes down regulation of VEGFR2 expression which in turn suppresses VEGF signalling pathway. This results in inhibition of excessive vessel branching by prevention of endothelial tip cell formation. Thus, this crosstalk suggests that for the inhibition of tumor progression and angiogenesis, simultaneous inhibition of both signalling pathways. Navicixizumab/HD105, a bispecific antibody blocks both VEGF/VEGFR and DLL4/Notch pathways in endothelial cells resulting in inhibition of proliferation and signalling. [39]

6. Mechanisms of Action of Bispecific antibodies in ailments other than cancer:

- Two factors dimerization: Emicizumab binds to both the activated coagulation factor IX and to factor X, facilitating the cascade reaction by mediating the activation of the factor X. This function is of coagulation factor VIII, which is deficient in haemophilia A patients. Phase 3 clinical trials HAVEN1, 2, 3 and 4 are ongoing. [40]
- Targeted Apoptosis: RG7386 simultaneously targets Fibroblast

activation protein (FAP) on cancer associated fibroblasts and Death receptor-5 (DR-5) on cancer cells leading to acidity driven hyper clustering of DR-5 and subsequently induces apoptosis in FAP positive tumor cells. Activation if DR-5 leads to formation of death inducing signaling complex followed by subsequent activation of caspase substrate 3,6 and 7 causing cell apoptosis. RG7386 showed potent tumor cells apoptosis in vitro and in vivo in preclinical tumor models with FAP positive stroma. [41]

- PK modulated receptor antagonists: Vobarilizumab targets IL6 via its IL6 receptor. It's one arm is anti-IL-6R nanobody linked to an anti-Human Serum Albumin (HSA) nanobody. The HSA binding is meant to increase the in vivo serum half life. IL6 is involved in stimulation of osteoclast differentiation and activation. Similar to Vobarilizumab, Ozoralizumab binds its one arm with TNF, neutralising it and other arm with Albumin to increase its in vivo serum half life. [15]
- Hormone mimetic action: RG-7992 is a bispecific antibody targeting Klotho beta protein and Fibroblast growth factor receptor-1 (FGFR1), mimicking the metabolic hormone FHF1. Phase 1 of clinical trial of RG-7992 has been completed. (NCT02593331)
- Two proteins/Two ligands inactivation: Many bispecific antibodies binding and inhibiting molecules are in clinical development for various cancer as well as non-cancer diseases. XBI1034020 binding and inactivating beta amyloid 40 and 42 has been described for the treatment of Alzheimers and is currently being studied in phase 1 of clinical trials (NCT01958060).ALX0761 for inflammatory diseases, RG7990 for Asthma, MEDI 7352 for Osteoarthritis, MEDI 0700 for Lupus arythromatosis, LY3114062 for rheumatoid arthritis targeting IL17A×IL17F, IL13×IL17, NGF×TNF, BAFF×b7RPI and TNF×IL17 respectively are currently in phase 1 of clinical trials. [15]
- Against Bacteria: Medi-3902 attacks Pseudomonas aeroginosa bacterium and neutralized it's defences. One arm of it binds to Psl antigen and the other arm binds to PcrV. PcrV and PRLR play an important role in acute and chronic infection caused by Pseudomonas aeroginosa. Psl is an exopolysaccharide that increases the availability of neutrophils in recognising and phagocytosis the bacterium and PcrV is a serotype independent type III secretion system virulence factor, that prevents release of bacterium factors capable of neutralising phagocytosis process. Patients infected generally were known to lack preexisting immunity and thus, couldn't generate a humoral response against these antigens. MEDI-3902 showed superior synergistic protection against Pseudomonas aeruginosa-induced murine pneumonia compared to parent mAb combinations. [42]
- Transmembrane transcytosis: The passage of large molecules across blood brain barrier is restricted due to the presence of tight junctions between endothelial cells in brain capillaries. Transport across bbb is allowed by specific binding to receptors such as TfR that internalised and release the ligand across the capillary endothelium. A bispecific antibody binding to TfR with one arm and BACE1 with other arm was demonstrated. BACE1 (Beta amyloid precursor protein cleavage enzyme) is a and enzyme that cleaves beta amyloid precursor protein and release soluble Amyloid beta into brain interstitium. By binding it, the bsAb ensures it's inhibition which leads to reduction in soluble beta amyloid levels in the brain preventing amyloid plaque formation. [43]
- Wnt Signalling: Wnt Signalling is an important event in the osteogenesis and bone formation that takes place during growth, bone homeostasis or fracture

repair. Sclerostin is a secreted factor produced by osteocytes that partly block Wnt Signalling by binding to LRP5 and LRP6 (Coreceptors expressed on surface of bone cells). Dickkopf-1 is another secreted factor that acts as Wnt antagonist by blocking the binding of Wnt proteins to LRP5 and LRP6. Monica et al demonstrated that dual inhibition of Sclerostin and DKK-1 by a bispecific antibody leads to synergistic bone formation in rodents and nonhuman primates. [44]

- bNAbs: Broadly neutralizing antibodies (bNAbs) against the HIV-1 envelope glycoprotein (Env) have been shown to potently suppress viremia in animal models of HIV-1 and humans. To ensure high potency without chance of mutant escape, targeting distinct epitopes required for the survival of virus is needed. For this, Bispecific neutralizing antibodies improving neutralisation activity are constructed. [45,46]

CONCLUSION

Bispecific antibodies have shown great therapeutic potential in oncological as well as non-oncological indications. The growing field of immunotherapy needs more understanding of mechanisms through which therapeutic efficacy can be obtained. We have attempted to discuss the mechanisms of working of bispecific antibodies by focusing on the bsAbs under investigation.

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How to cite this article: Habeeb A, Hajree S. Bispecific antibodies: progress and application. Int J Health Sci Res. 2018; 8(1):259-272.

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