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Review

New insights into affinity proteins for HER2-targeted therapy: Beyond trastuzumab

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2) is known as a potential target for both cancer treatment and diagnosis. One of the most interesting HER2-targeted therapeutics is an affinity protein which selectively recognizes and binds to a defined target. Trastuzumab is a monoclonal antibody which has been approved as the first affinity proteins for treatment of some HER2-positive cancers including breast cancer. Despite initial response to trastuzumab, the majority of patients with metastatic HER2-positive breast cancer still show resistance to the therapy. Recently, various anti-HER2 affinity proteins, including antibodies, antibody fragments (*e.g.*, Fab and scFv) and other protein scaffolds (*e.g.*, affibody and DARPin), alone or fused/conjugated with therapeutic agents (*e.g.*, proteins, drugs and radioisotopes) have been developed to overcome the trastuzumab resistance. Here, we review these engineered affinity proteins which are either clinically approved or under evaluation. Modern technologies and future prospects for their clinical applications in cancer treatment are also discussed.

1. Introduction

1.1. Affinity proteins: a brief overview of their structures, types and generation

Affinity proteins can selectively recognize and bind to target molecules and, therefore, are invaluable agents for diagnostics and therapeutics. In the early 1900s, the side-chain theory or "magic bullet" was proposed that molecules could be structurally designed to recognize or destroy specific microbes or tumor cells [1]. Since then, many targeting agents have been developed and among them antibodies are the most successful and widespread. Antibodies or immunoglobulins (Ig) are Y-shaped glycoproteins produced by B cells to recognize, neutralize, and even destroy cell attackers, such as pathogens or cancerous cells [2].

Basically, full-size antibodies (*i.e.*, IgG) are large molecules (~150 kDa) consisting of variable (V) and constant (C) regions for both light (L) chain (comprising the CL and VL domains) and heavy (H) chain (comprising the CH1, CH2, CH3, and VH domains) (Fig. 1) with a complex glycosylation pattern and several disulfide bonds, resulting in challenging and expensive manufacturing process [3]. Large-scale production of monoclonal antibodies requires proper selection of cell lines, cell adaptation to culture conditions, and bioreactor design [4]. In

Abbreviation: ABD, Albumin-binding domain; ADC, Antibody-drug conjugate; ADCC, Antibody-dependent cellular cytotoxicity; ADEPT, Antibody-directed enzyme prodrug therapy; AIF, Apoptosis-inducing factor; Akt, Protein kinase B; CCL5, Chemokine (C–C motif) ligand 5; CDC, Complement-dependent cytotoxicity; CEA, Carcinoembryonic antigen; COX-2, Cyclooxygenase-2; CSF, Colony-stimulating factor; CXCR4, CXC chemokine receptor 4; DARPin, Designed ankyrin repeat protein; DM1, Mertansine; dsFv, Disulfide-stabilized Fv antibody fragment; DT, *Diphtheria* toxin; ECD, Extracellular domain; EGFR, Epidermal growth factor receptor; EpCAM, Epithelial cell adhesion molecule; FDA, Food and Drug Administration; ETA, Shortened version of *Pseudomonas* exotoxin A; Fab, Antigen-binding fragment; Fc, Fragment crystallizable; GFP, Green fluorescent protein; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GrB, Granzyme B; HER2, Human epidermal growth factor receptor 2; HP-RNase, Human pancreatic RNase; IFN, Interferon; Ig, Immunoglobulin; IL, Interleukin; kDa, Kilodalton; MAPK, Mitogen-activated protein kinase; miniSOG, mini Singlet Oxygen Generator; MOA, Mechanism of action; mTOR, Mechanistic target of rapamycin; NK cell, Natural killer cell; PCI, Photochemical internalization; PE, *Pseudomonas* exotoxin A; PEG, *Polyethylene glycol*; PIK3, Phosphoinositide-3-kinase; RAF, Rapidly Accelerated Fibrosarcoma; RAS, Rat sarcoma; RAT, Ricin A chain; RNase, Ribonuclease; scFv, Single chain variable fragment; siRNA, Small interfering RNA; SrtA, Sortase A; STAT3, Signal transducer and activator of transcription 3; TCR, T-cell receptor; T-DM1, Ado-trastuzumab emtansine; TKIs, Tyrosine kinase inhibitors; TNF, Tumor necrosis factor; TRAIL, TNF related apoptosis inducing ligand; VEGF, Vascular endothelial growth factor; VLP, Virus-like particle

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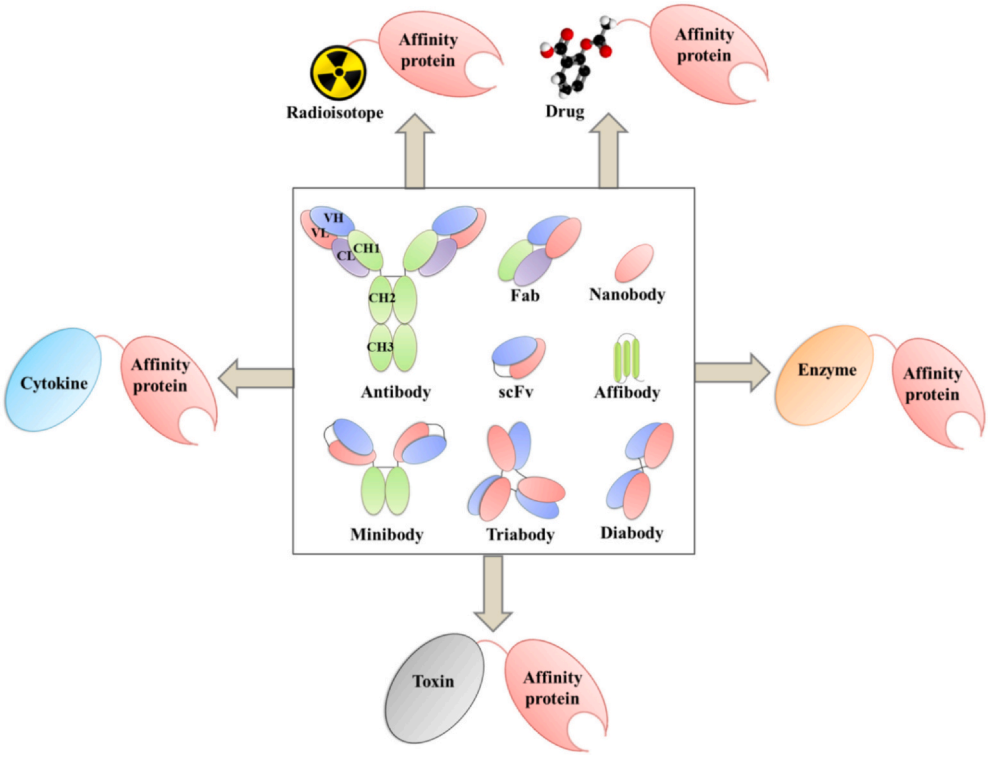

Fig. 1. Different types of affinity protein and different agents targeted by affinity proteins. Proteins having ability to selectively recognize and strongly bind to a given target molecule are affinity proteins, including antibody, minibody, triabody, diabody, Fab, scFv, nanobody and affibody. A variety of agents including proteins (*e.g.*, enzyme, toxin and cytokine), drugs and radioisotopes can be linked to an affinity protein (shown as dark brown symbol) for diagnosis and therapeutic applications.

addition, full-size antibodies often have a limited molecular access to certain tissues (*e.g.*, tumor tissues) due to their relatively large sizes [5]. These limitations entail development of smaller antibody fragments as reliable alternatives, including antigen-binding fragments (Fab), single-chain variable fragments (scFv), diabodies, traibodies, minibodies and single domain antibodies (*e.g.*, nanobodies) (Fig. 1). These antibody fragments retain antigen-binding sites of the full-size antibodies, but their manufacturing processes can be significantly less complicated and more cost-effective. For example, scFv are non-glycosylated small-size polypeptides (~26–28 kDa), which can be actively expressed in both prokaryotic and eukaryotic host systems [6]. Bacterium *Escherichia coli* is a common host for effective production of antibody fragments with high titers in g/L levels [7]. However, the efficiency of antibody fragment expression is dependent on a variety of biological and bioprocessing factors, such as amino acid sequence, polypeptide size, and cultivation condition, such that the expression strategy must be customized for individual polypeptide [8,9]. ScFv have been functionally expressed in *E. coli* using different protein expression and localization systems, including those secreted extracellularly [10] and those residing in the outer membrane [11], the periplasm [12], the inner membrane or the cytoplasm [13].

In light of successful development and application of antibody fragments, alternative protein scaffolds for molecular recognition have also been explored. Most of these new protein scaffolds have similar promising properties, such as small size, simple structure as a single-chain polypeptide, high and disulfide-bond independent stability, all of which potentially facilitate their large-scale production in bacterial expression systems, clinical applications, and even derivation of multi-specific molecules [14]. Over the past years, approximately 50 non-immunoglobulin scaffolds, including adnectins, affibodies, anticalins, designed ankyrin repeat proteins (DARPin), protease inhibitors, and peptide aptamers, have been explored, leading to various applications as the next-generation immuno-therapeutics [15]. For example, DARPin are genetically modified antibody mimetic proteins derived from ankyrin proteins and have been applied as both a useful investigational tool (*e.g.*, crystallization chaperones and biosensors) and an effective protein scaffold for diagnostic and therapeutic applications [16]. DARPin usually consist of four or five repeat motifs proteins (~14 or 18 kDa, respectively) with an extremely high stability and aggregation resistance as they lack any β-sheet structure [17]. DARPin can be expressed in the cytoplasm of *E. coli* at high concentrations (> 10 g/L) in a soluble form [18]. Due to their high binding specificity and affinity, high stability and solubility (> 100 g/L), structural flexibility enabling construction of different multi-specific molecules, conjugates (*e.g.*, polyethylene glycol (PEG)) and fusions (*e.g.*, human serum albumin), as well as cost-effective production, DARPin are widely explored as drug compounds [17]. On the other hand, affibodies represent one of the most effective protein scaffolds for medical diagnostics, therapeutics, and biotechnological applications. Affibodies are small-size molecules (~7 kDa; 58 amino acids) derived from staphylococcal protein A, consist of three alpha helices, have rapid folding kinetics, and lack disulfide bonds, making them highly soluble and stable [19]. Specific binding properties of affibodies are obtained by proper design of the 13 surface-exposed amino acid residues in helix one and two [20]. Because of the small size, affibodies can be efficiently synthesized by either solid-phase peptide synthesis or bacterial expression systems [20].

In addition to their direct therapeutic and diagnostic applications, affinity proteins can be linked or conjugated to various other therapeutic agents, including proteins (*e.g.* enzyme, cytokine and toxin), drugs, radioisotopes and particles (*e.g.*, liposomes, nanoparticles, viral and bacterial particles) (Fig. 1), for more effective medical treatment. With the direction of the affinity protein moiety, these fusions (*i.e.*, when an affinity protein is joined to other protein(s) *via* an amino acid linker) or conjugates (*i.e.*, when an affinity protein is attached to other non-protein agents by covalent or non-covalent bonds) can be selectively delivered to a specific target, such as cancer cells. These affinity protein conjugates and fusions can be derived using various genetic, enzymatic and chemical methods [21]. Affinity proteins have certain functional groups, such as lysine, cysteine, which can be used for random or site-specific conjugation. Random conjugation can lead to a heterogeneous mixture of conjugates, while site-specific conjugation generates homogenous conjugates. Large affinity proteins, such as intact antibodies, are more suitable for site-specific conjugation as they have too many functional groups and fragment crystallizable (Fc)

regions for engineering [22].

1.2. Human epidermal growth factor receptor 2 (HER2) as a therapeutic target

Human epidermal growth factor receptor 2 (HER2) (also known as p185 or ErbB2) is a human protein involved in normal cell growth and can act as a marker in certain types of cancer cells. HER2 and its relatives HER1 (also known as EGFR), HER3 and HER4 belong to the ErbB family [23]. These receptors are structurally related transmembrane proteins consisting of an extracellular domain (ECD) with four subdomains (I-IV), a transmembrane domain, and a cytoplasmic domain. Receptor dimerization or receptor pairing is necessary for signal transduction *via* the ErbB family [24]. While HER2 is the only member of the ErbB family without an identified ligand, it acts as a preferential partner for heterodimer formation with other family members [25]. Unlike the other ErbB family members, the ECD of HER2 is consistently in an extended conformation that promotes the exposure of the dimerization arm, located in subdomain II, for protein interaction and heterodimer formation. Furthermore, heterodimers containing HER2 generate the strongest intracellular signal among receptor pairs formed by the ErbB family members [26]. The main signaling pathways mediated by HER2 involve rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase (MAPK) pathways and phosphoinositide-3-kinase (PIK3)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) kinase pathways, which regulate growth, proliferation, differentiation, motility, and adhesion of normal cells [27].

Alterations of HER2 functions have been discovered in cancer cells. HER2 gene amplification (*i.e.* the presence of multiple gene copies), overexpression, and mutation have been identified in tumor progression and tumor metastasis. Amplification of the HER2 gene and deregulation of the transcription can result in overexpression of HER, which increases cancer cell proliferation, invasion, and anti-apoptosis [28]. In particular, breast cancer cells can express HER2 at levels 10–100 fold higher than normal epithelial cells [29]. HER2 overexpression usually occurs with truncated forms of HER2 (p95-HER2) resulting from the shedding of ECD *via* proteolytic cleavage, alternative start sites for translation or alternative patterns for splicing of mRNA precursors [30]. Also, HER2 overexpression and amplification can lead to poor prognosis of many kinds of cancer, including breast, gastric, ovarian, prostate, esophageal, and endometrial [31]. The formation of p95-HER2 fragments is often related with poor prognosis, metastasis and resistance to anti-HER2 treatments [32].

The importance of HER2 in tumorigenesis is not just limited to its proliferative effects. Some studies showed that HER2 can be a transcriptional factor for other tumor markers The presence of HER2, both full-length and truncated forms, in the nucleus has been evidenced in many kinds of cancer cell lines and primary tissues [33,34]. Internalization of HER2 through endocytosis and its translocation into the nucleus *via* interaction with the transport receptor (*i.e.*, importin β1) and the nuclear pore protein (*i.e.*, Nup358) have been previously proposed [35]. In the nucleus, HER2 might contribute in phosphorylation of other transcriptional factors and histone modification regulating gene expression. Further studies are still required to understand the exact mechanism of nuclear localization of HER2 and its transcriptional regulatory activity. Several studies noted the co-activator role of nuclear HER2 for signal transducer and activator of transcription 3 (STAT3), contributing in progestin-driven activation of cyclin D1 promoter [36,37].On the other hand, the interaction of HER2 and DNA was observed. A recent investigation applied ChIP-exo and ChIP-seq methods for genome-wide analysis of HER2 binding sites in two breast cancer cell lines and found over 2000 HER2-bound regions [38]. Furthermore, nuclear HER2 has been shown to directly act as a transcription factor for some tumor marker genes, including cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and CXC chemokine receptor 4 (CXCR4). For example, HER2 can bind to the promoter region of COX-2 and up-regulate expression of this enzyme which plays a role in tumor progression, immune evasion and resistance to chemotherapy [34,39]. Overexpression of HER2 is related with COX-2 expression particularly in metastatic cancers, resulting in poor prognosis [34]. It has been well documented that HER2 increases the expression and inhibits ligand-induced degradation of CXCR4 which may be involved in motility and invasion of breast cancer cells to other organs [40]. HER2-enhanced CXCR4 expression is also known to be associated with poor prognosis [41]. On the other hand, VEGF plays an important role in tumor angiogenesis and HER2 signaling induces VEGF expression in cancer cells [42]. Understanding such molecular interaction has led to an effective treatment of brain metastases spreading from HER2-amplified breast cancer through a combined therapy targeting VEGF and HER2 [43].

Considering the relation with poor clinical outcomes in HER2-positive breast cancer patients and emergence of resistance to anti-HER2 treatments [33,44], nuclear HER2 (*i.e.*, p185 and p95) can be a novel biomarker for tumor prognosis and a potential target for development of more effective therapeutic agents (*e.g.*, by selective blockage of HER2 nuclear translocation and activity) [45]. In one study, a HER2 nuclear localization signal mutant was developed to demonstrate that blocking nuclear HER2 localization could inhibit cancer cell proliferation and drug resistance [46]. Targeting nuclear proteins is usually challenging and, until now, there is no efficient drug that can specifically inhibit nuclear HER2 localization and function. However, some anti-HER2 agents (*e.g.*, antibodies and small molecule inhibitors) have been shown to reduce nuclear translocation of HER2 [47]. Additionally, suppression of HER2 cleavage (*i.e.*, p95) by proteinase inhibitors and inhibition of the expression of nuclear transport receptors by siRNAs might be effective [48].

In light of the key biological role of HER2 in tumorigenesis, HER2 can be a potential target for both cancer treatment and diagnosis. Anti-HER2 drugs and biopharmaceuticals are currently applied for treating HER2-positive gastric cancer in several clinical trials [49]. There are reports about the effectiveness of HER2-targeted treatment in the lung adenocarcinoma patients with HER2-mutations [50], as well as the effectiveness and safety of ErbB inhibitors in treating patients with castrate-resistant prostate cancer [51].

Many therapeutic agents including affinity proteins (*e.g.*, antibody) and small-molecule inhibitors (*e.g.*, tyrosine kinase inhibitors (TKIs)) have been developed to target HER2 receptor. TKIs can translocate through the cell membrane and inhibit tyrosine kinase activity of the cytoplasmic domain of receptor, resulting in inhibiton HER2 signaling pathways and subsequent suppression of cancer cell proliferation [52]. In addition to the interaction with the ECD of HER2, antibodies can inhibit tumor growth *via* other mechanisms involving immune responses. Development of small-molecule inhibitors can be less complex and expensive than antibodies. However, due to a higher specificity and effectiveness, antibodies can be easier of getting clinical approval than chemically synthesized small-molecules [53].

In October 1998, US Food and Drug Administration (FDA) approved trastuzumab (Herceptin®) as the first HER2-targeted therapy for treating HER2-positive breast cancer. While patients with metastatic HER2-positive breast cancer showed an initial response to trastuzumab, most of them developed resistance to the therapy after one year of the treatment [54]. To overcome the trastuzumab resistance, various other HER2-targeting agents have been developed. Over the past decades, several anti-HER2 affinity proteins (*e.g.*, full-size antibody (IgG), Fab, scFv, nanobodies and affibodies) fused/conjugated with therapeutic agents (*e.g.*, proteins, drugs and radioisotopes) have been developed for HER2-targeted therapy. Here, we review these engineered affinity proteins as potential HER2-targeted therapeutics, which are either clinically approved or under evaluation. Modern technologies and future prospects for their applications in cancer treatment are also discussed.

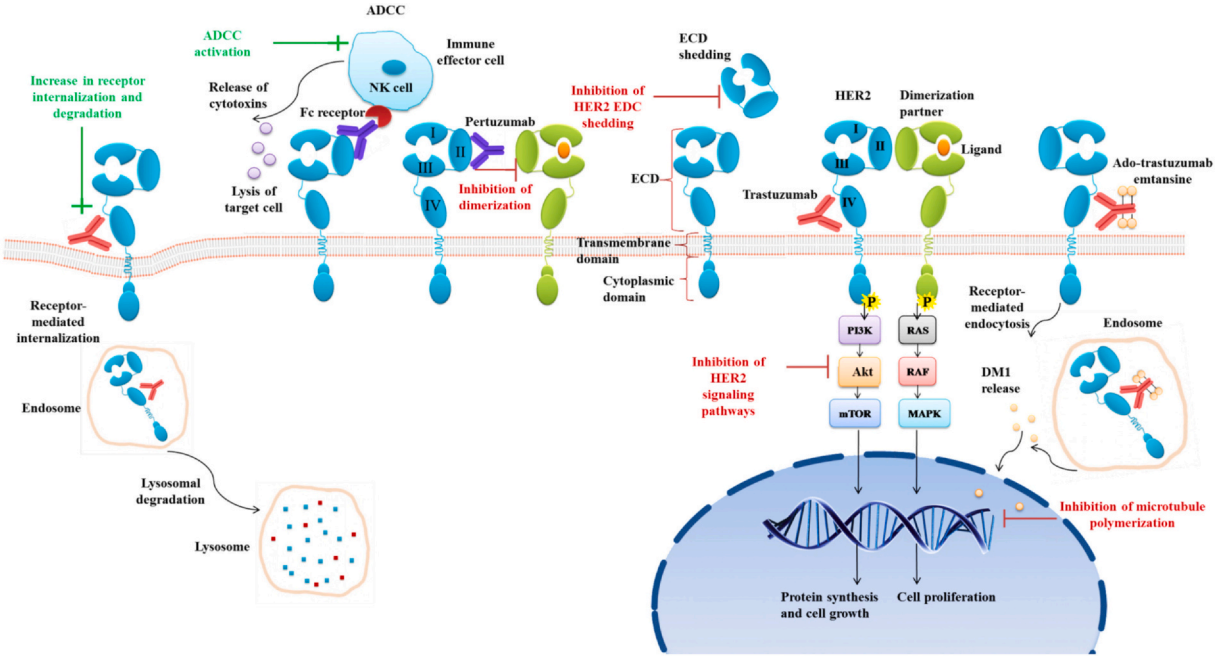

Fig. 2. The proposed MOAs for FDA-approved anti-HER antibodies. Trastuzumab binds to HER2 particularly subdomain IV. This antibody can stimulate activation of antibody-dependent cellular cytotoxicity (ADCC). Trastuzumab prevents phosphorylation of the cytoplasmic domain of HER2, resulting in inhibition of the PI3K/Akt/mTOR and RAS/RAF/MAPK signaling pathways. Furthermore, trastuzumab inhibits shedding of HER2 ECD (p95) through proteolytic cleavage. Additionally, trastuzumab can down regulate HER2 through increase in receptor internalization and degradation. Pertuzumab binds to subdomain II of HER2. This antibody mainly inhibits dimerization of HER2. Pertuzumab can also kill cancer cells through activation of ADCC. T-DM1 internalizes into cancer cell through binding to HER2 and undergoes intracellular proteolytic degradation leading to release of its cytotoxic moiety, DM1. The cytotoxic agent, DM1, enters into nucleus of the tumor cell and inhibits microtubule polymerization, thereby inducing a cell-cycle arrest and apoptosis. T-DM1, like trastuzumab, inhibits HER2-mediated signal transduction, binds to Fc receptor of immune effector cells and activates ADCC.

2. Clinically approved therapeutics that target HER2 for treating breast cancer

Currently, there are four HER2-targeted antibodies approved by FDA, *i.e.* trastuzumab, pertuzumab, Ado-trastuzumab emtansine (T-DM1), and Fam-trastuzumab deruxtecan-nxki, for treating patients with HER2-overexpressing breast cancer and their potential mechanisms of action (MOAs) are described in Fig. 2.

2.1. Trastuzumab

Trastuzumab (Herceptin®), a humanized monoclonal antibody (IgG), was approved by FDA as the first HER2-targeted therapeutics for treating patients with HER2-positive breast cancer. Trastuzumab binds on the epitope in the extracellular subdomain IV of HER2. Although precise molecular and cellular mechanisms through which trastuzumab inhibits the growth of HER2-positive cancer cells are not fully understood, several hypothetic MOAs are proposed.

One of them is the activation of antibody-dependent cellular cytotoxicity (ADCC) which involves the stimulation of immune system effector cells, mainly natural killer (NK) cells [55]. NK cells recognize and bind to the Fc region of an antibody (*i.e.*, trastuzuamb in this case) which also has an affinity to a specific antigen on the surface of target cancer cells, leading to the secretion of cytotoxic agents from NK cells for lysis of target cancer cells. It was reported that, upon the treatment with trastuzumab, the number of both NK cells and proteins that are toxic to cancer cells (*e.g.*, Granzyme B) increased in surgical tumor specimens of mastectomy patients [56].

Furthermore, trastuzumab mediates down-regulation of HER2 receptor, resulting in a decrease in the number of HER2 receptors on the surface of target cells, through internalization and degradation of antibody-receptor complexes. The attachment of trastuzumab to HER2 potentially promotes HER2 ubiquitination and degradation by recruiting c-Cbl, an ubiquitin ligase, to its binding site at tyrosine 1112 [57]. HER2 recycling is a dynamic and complex process and its exact mechanism is not fully understood. It should be noted that HER2 internalization and ubiquitination can also impact the therapeutic efficacy of trastuzumab and other anti-HER2 agents. For instance, TKIs which increase ubiquitination and endocytosis of antibody-receptor complexes have been shown to improve the effectivness of anti-HER2 agents particularly antibody-drug conjugates (ADC) such as ado-trastuzumab emtansine and fam-trastuzumab deruxtecan-nxki [58]. Several studies reported that anti-HER2 treatment-resistant cell lines contained higher intracellular HER2 than membrane-localized receptor [59]. Some trafficking proteins (*e.g.*, sortilin-related receptor 1 and caveolin-1) contribute to HER2 oncogenesis and resistance to anti-HER2 treatments [59]. It was shown that HER2 is concentrated in caveolae (*i.e.*, a special type of lipid raft) membrane domains and caveolin-1 is involved in cellular trafficking of the receptor [60]. Down-regulation of Caveolin-1 reduced HER2 endocytosis and enhanced its stability and availability on the surface of cancer cells, resulting in improved effectiveness of trastuzumab in patient-derived tumor xenografts [61].

It was also proposed that trastuzumab inhibits the PI3K/Akt and MAPK signaling pathways *via* reducing PI3K and Akt kinase activities [62]. On the other hand, the binding of trastuzumab to HER2 increases the activity of phosphatase and tensin homolog (PTEN), a tumor suppressor protein which inhibits the PI3K/Akt pathway, *via* blocking the Src tyrosine kinase signaling [63]. Though trastuzumab-containing therapies, either alone or in combination with other agents, have been shown to increase both response rate and survival rate, drug resistance is a major challenge for breast cancer treatment [64]. Further efforts are still needed toward designing more specific and effective therapeutic agents for treating patients with trastuzumab-resistant breast cancer.

In February 2019, Herceptin Hylecta®, a novel formulation of trastuzumab in combination with hyaluronidase was approved by FDA for

subcutaneous use in patients with HER2-positive breast cancer. Based on preclinical studies, hyaluronidase, an enzyme that depolymerizes hyaluronic acid, a main component of the extracellular matrix of connective tissues, can improve the absorption of trastuzumab when administered subcutaneously. Phase III clinical studies revealed that subcutaneous trastuzumab showed asimilar efficacy and safety to intravenous formulation [65,66]. Furthermore, patients preferred subcutaneous over intravenous formulation as the former required less time to be administrated (*i.e.* 2–5 min *versus* 30–90 min). Unlike intravenous trastuzumab, subcutaneous formulation does not need a loading dose and can be administrated in a fixed-dosage regimen regardless of bodyweight [67].

2.2. Pertuzumab

Pertuzumab (Perjecta®), a humanized monoclonal antibody (IgG) interacting with extracellular subdomains I, II and III, is one of the novel generation of HER2 antibodies known as dimerization inhibitors [25]. In June 2012, FDA approved pertuzumab for use with docetaxel (Taxotere) and trastuzumab for treating HER2-positive metastatic breast cancer [68]. The X-ray crystallographic structure of HER2-pertuzumab complex suggests that pertuzumab can sterically inhibit HER2 dimerization with other ErbB family [25]. *In-vitro* studies on prostate and breast cancer cell lines also showed that pertuzumab can disrupt the formation of HER2 hetrodimer with HER3 or HER1, a potential mechanism for developing trastuzumab resistance of cancer cells, in the presence of high-level heregulin [69]. Similarly, it was shown *in vitro* that pertuzumab, but not trastuzumab, inhibited HER2 signaling following HER3 activation [69]. Furthermore, both full-size antibody and Fab fragment inhibited the growth of cancer cells *in vitro* and *in vivo*, indicating that the presence of Fc, contributing to ADCC, might not be critical for inhibition of cancer cell growth [69]. Preclinical evidence shows that the formation of HER2/HER3 heterodimers is a better indicator of the response to pertuzumab than HER1, HER2 or HER3 expression or phosphorylation levels of the receptors [70]. Pertuzumab has a lower toxicity level without a compromised treating effectiveness compared to trastuzumab, as the cellular cytotoxicity associated with antibody is not necessarily proportional to its treating effectiveness [71].

2.3. Ado-trastuzumab emtansine

Ado-trastuzumab emtansine (Kadcyla®) was approved in February 2013 by FDA for treating patients with metastatic HER2-positive breast cancer who failed to receive an effective treatment from trastuzumab, either alone or in combination with a taxane. *In vitro*, ado-trastuzumab emtansine (T-DM1) was active against both trastuzumab and lapatinib-resistant cell lines and tumors [72]. T-DM1, composed of trastuzumab and a cytotoxic agent mertansine (DM1), is the first ADC with a non-reducible thioether spacer for clinical trials. While different trastuzumab-DM1 conjugates were derived using various disulfide spacers or linkers, the ADC with a non-reducible thioether linker exhibited more effectiveness and less toxicity than those bearing a reducible spacer [73]. Like trastuzumab, T-DM1 inhibits HER2-mediated signal transduction and activates ADCC through binding to the Fc receptor of NK cells. After internalization through binding to HER2 overexpressed on the surface of cancer cells and receptor-mediated endocytosis, T-DM1 undergoes intracellular proteolytic degradation within endosome, leading to the release of its cytotoxic moiety DM1 which inhibits microtubule polymerization and consequently induces the cell cycle arrest and apoptosis [74]. Based on promising results of T-DM1 in mouse models of brain metastases, this ADC entered clinical phases [75]. A recent cohort study (*i.e.*, KAMILLA trial) suggested the efficacy and safety of T-DM1 in patients with HER2-positive breast cancer brain metastases who previously failed to the treatment with trastuzumab [76]. A significant reduction in the size of brain target lesions was achieved in 42% of patients during the treatment with T-DM1. A 5.5-month median progression-free survival (95% CI 5.3–5.6) was observed and the median overall survival was 18.9 (95% CI 17.1–21.3) months.

2.4. Fam-trastuzumab deruxtecan-nxki

Fam-trastuzumab deruxtecan-nxki or DS-8201a (Enhertu®) is another ADC composed of trastuzumab and an exatecan derivative (deruxtecan) as a potent inhibitor of topoisomerase I, which induces and stabilizes DNA single-strand breaks leading to apoptosis in HER2-overexpressing cancer cells [77]. Based on the promising anti-tumor efficacy (*e.i.*, being effective in xenografted animals irrespective to HER2 expression level of tumors), a phase I study was conducted to assess the safety and tolerability of DS-8201a in patients with advanced solid malignomas [ClinicalTrials.gov identifier: NCT02564900]. An objective response rate of 59% and an acceptable safety profile were reported for patients with HER2-overexpressing breast cancer pretreated with T-DM1 [78]. In patients with HER2-low expressing breast cancer, DS-8201a was also well tolerated and the overall response rate was 55% [79]. A phase II trial was conducted to evaluate efficacy and pharmacokinetic of DS-8201a in subjects with HER2-expressing advanced breast cancer previously treated with T-DM1 [ClinicalTrials.gov identifier: NCT03248492]. DS-8201 demonstrated meaningful efficacy (*i.e.*, achieving an overall response rate of 60%) and reasonable tolerability in heavily pretreated HER2-positive metastatic breast cancer patients [80]. Two ongoing phase III trials have been conducting to compare efficacy of DS-8201a with physicians' choice in patients with HER2-positive [ClinicalTrials.gov identifier: NCT03523585] and HER2-low expressing [ClinicalTrials.gov identifier: NCT03734029] advanced breast cancer. According to the promising results of phase II trial, DS-8201a was granted accelerated approval by FDA on December 20, 2019 for the treatment of unresectable or metastatic HER2-positive breast cancer patients previously received two or more prior anti-HER2-targeted therapies [81].Novel HER2-targeted agents under evaluation

2.5. Antibodies, antibody fragments and other protein scaffolds

Currently, several novel anti-HER2 antibodies, antibody fragments and other protein scaffolds are under investigation, both in preclinical and clinical (see Table 1) stages, including Fabs, scFvs, nanobadies, DARPin, bispecific and trispecific antibodies.

2.5.1. Margetuximab

Margetuximab is an Fc-engineered chimeric monoclonal antibody (IgG) preserving the antigen-biding properties of trastuzumab, but with an optimized Fc region to modify IgG's interaction with activating (*e.g.*, RIIIA/CD16A) and inhibitory (*e.g.*, RIIB/CD32B) Fc-gamma receptors on immune effector cells. There are two variant of activating Fc-gamma receptors, with high (158 V) or low (158F) binding affinity to the Fc of IgG1. Fc-gamma receptor polymorphisms correlate with the clinical response of breast cancer patients treated with trastuzumab. Patients harboring CD16A-158 V/V genotype or CD32A-131H/H genotype or both show a better progression-free survival rate than those with the corresponding 158 (V/F or F/F) or 131 (H/R or R/R) genotypes [82]. Margetuximab has a engineered Fc region with an increased binding affinity to Fc-gamma activating receptors, including low affinity variant (158F), and a reduced binding affinity to Fc-gamma inhibitory receptors on immune cells. Modification of the Fc domain can potentially mediate a stronger ADCC activity of trastuzumab, even against trastuzumab-refractory or low HER2-expression cancer cells, regardless of the Fc-gamma receptor variants [83]. Phase I clinical trial of margetuximab in patients with HER2-positive carcinoma showed a high tolerance and promising effectiveness, particularly for patients with breast cancer and gastroesophageal cancer who had failed treatment with trastuzumab [84]. Currently, a phase II trial of combination margetuximab and pembrolizumab, an anti-PD1 (programmed death-1 receptor)

Table 1
Some of antibodies antibody fragments and other affinity protein for HER2-targeted therapy under clinical evaluation.

Agent	Type	Target(s)	Status	Reference
Margetuximab	Fc-engineered chimeric IgG	HER2	In phase III for HER2-positive metastatic breast cancer	[85]
Trastuzumab-GEX	Glycol-optimized IgG	HER2	In phase I for advanced or metastatic HER2-positive solid tumors	[89]
ZW25	Bispecific antibody	HER2 (subdomain II and IV)	In phase II for advanced HER2-positive gastroesophageal adenocarcinoma	[91]
MCLA-128	Glycoengineered bispecific antibody	HER2/HER3	In phase I/II for advanced HER2-positive solid tumors	[95]
PRS-343	Bispecific antibody/anticalin® protein	HER2/CD137	In phase I for advanced HER2-positive solid tumors	[97]
GBR 1302	Bispecific antibody (BEAT)	HER2/ CD3	In phase I/II for advanced HER2-positive breast cancer	[103]
MP0274	Trispecific DARPin®	HER2 (subdomain II and IV)/ albumin	In phase I for advanced HER2-positive solid tumors	[105]
MBS301	Glyco-engineered bispecific antibody	HER2 (subdomain II and IV)/ albumin	In phase I for advanced HER2-positive solid tumors	[106]
KN026	Bispecific antibody (CRIB)	HER2 (subdomain II and IV)	In phase II for HER2-positive breast and gastric cancers	[107]

antibody, for advanced HER2 positive gastric or gastroesophageal cancer is conducted at 25 sites across North America and Asia [ClinicalTrials.gov identifier: NCT02689284]. In addition, a phase III trial started in 2015 to evaluate the efficacy of combining margetuximab *versus* trastuzumab with the single agent chemotherapy in patients with advanced HER2-overexpressing breast cancer who had previous ineffective treatment with other anti-HER2-targeted therapies [ClinicalTrials.gov identifier: NCT02492711]. The primary results of this ongoing but no longer recruiting patients trial showed an improved progression-free survival in patients received the combination of margetuximab plus chemotherapy compared to trastuzumab plus chemotherapy [85]. Based on these results, margetuximab biologics license application (BLA) has been submitted to FDA for the treatment of patients with metastatic HER2-positive breast cancer in combination with chemotherapy [86].

2.5.2. Trastuzumab-GEX

Trastuzumab-GEX (TrasGEX®), a glycol-optimized version of trastuzumab, is derived by glyco-engineered human suspension cell lines, the GlycoExpress platform [87], which enables the production of therapeutic proteins with proper sialylation, galactosylation, fucosylation, and branching, and without non-human sugar structures and glycosylation patterns. Trastuzumab-GEX is designed to preserve the antigen-biding properties of trastuzumab and improve ADCC-mediated antitumor efficacy for all CD16A genotypes [87]. Also, the use of human cell lines as the expression host will minimize common immunogenic reactions against non-human host components [87]. A long-term remission induced by trastuzumab-GEX and an approximate 10 to 140 fold improved ADCC was observed in a female patient with advanced HER2-positive colorectal cancer and CD16A 158 F/V for whom prior available *treatments have failed* [88]. A phase I clinical trial of trastuzumab-GEX was performed in patients with metastatic or locally advanced HER2-overexperssing solid tumors who had no effective standard treatments and showed that the engineered antibody was well tolerated with a promising activity in 50% of patients and the therapeutic effectiveness was irrespective of the Fc-gamma receptor genotype [89].

2.5.3. ZW25

ZW25, constructed using Azymetric technology, is a bispecific antibody that can simultaneously bind to two different and non-overlapping epitopes, II (*i.e.*, binding site of pertuzumab) and IV (*i.e.*, binding site of trastuzumab) subdomains of HER2 [90]. Based on preclinical studies, unique mode of ZW25 binding to HER2 enhances the receptor clustering and internalization which inhibits HER2 activity and signaling [90]. Thanks to its higher binding potential (*i.e.*, having two binding sites) to HER2-expressing cancer cells, ZW25 can bind more efficiently to the target cells leading to more immune effector cell (*e.g.*, NK) recruitment and thus an improved effector function-mediated cytotoxicity (*e.g.*, ADCC) compared with monospecific antibodies [90]. A phase I clinical trial of ZW25 was conducted in patients with metastatic or locally advanced HER2-overexperssing cancer who had progressed after standard therapies (*e.g.*, trastuzumab, pertuzumab, and T-DM1) and showed that ZW25 was well tolerated with a promising antitumor activity across a range of HER2-expressing cancers particularly in gastroesophegeal cancer (44% objective response rate) [91]. Currently, a phase II clinical trial is performed to evaluate the safety and efficacy of ZW25 added to chemotherapy in patients with HER2-expressing advanced gastroesophageal adenocarcinoma [ClinicalTrials.gov identifier: NCT03929666]. Based on promising results of the phase I, FDA has recently granted fast track designation to ZW25 as a treatment for patients with HER2-overexpressing gastroesophageal adenocarcinoma [92].

2.5.4. MCLA-128

MCLA-128 (Zenocutuzumab) is a bispecific humanized IgG1 antibody that simultaneously targets HER2 (*i.e,* subdomain I) and HER3 (*i.e,* subdomain III) [93]. This agent can significantly inhibit binding of neuregulin (NRG1; HER3 ligand) to HER3 and subsequent formation of HER2/HER3 heterodimers and activation of signaling pathways (*e.g.*, PIK3/Akt), and as a result, MCLA-128 can overcome HER3-mediated resistance to HER2-terageted therapies *in vitro* and *in vivo* [93]. MCLA-128 also inhibits the growth of tumor cells *via* an improved ADCC as it is glycol-engineered to be a low fucose antibody using GlymaxX technology [94]. Based on promising preclinical results, a phase I/II clinical trial was conducted to assess the safety and anticancer activity of MCLA-128 in patient with HER2-positive solid tumor and it was showed that it was very well tolerated with a promising antitumor activity [95]. A phase II clinical trial has been conducting to evaluate efficacy of MCLA-128 in patients with NRG1 fusion-positive tumors [96].

2.5.5. PRS-343

RPS-343 is a bispecific anti-CD137 /anti-HER2 protein consisting of an engineered IgG4 variant of trastuzumab and an anticalin, a synthetic antigen-binding non-immunoglobulin scaffold protein derived from lipocalins, that binds to CD137, a member of tumor necrosis factor (TNF) receptor family mainly expressed on activated T cells [97]. This agent can simultaneously bind to HER2-expressing tumor cells and CD137-expressing T cells which promotes CD137 clustering and generates a strong co-stimulatory signal to T cells leading to the cytotoxic T cell-mediated lysis of HER2-positive cancer cells [98]. RPS-343 exhibited an effective co-stimulation of T cells *in vitro*, a significant inhibition of tumor growth in humanized mice model and a good safety profile in animal model [97]. Considering the promising results of preclinical studies, recently a phase I clinical trial has been conducted to evaluate safety and efficacy of RPS-343 in patients with HER2-positive advanced solid tumors [ClinicalTrials.gov identifier: NCT03330561]. In addition to reasonable efficacy and safety profile of PRS-343, post-treatment

tumor biopsies in patients treated with this bispecific anti-CD137 /anti-HER2 protein showed a significant increase in cytotoxic T cell (*i.e,* $CD8^+$) numbers indicating CD137 clustering and activation on T cells [99].

2.5.6. GBR 1302

GBR 1302 is a bispecific antibody developed based on BEAT® platform to simultaneously target HER2-expressing tumor cells and CD3-expressing T cells, thus killing the antibody coated tumor cells by cytotoxic T cells [100]. BEAT molecule consists of three chains: a light chain, a heavy chain and a scFv-CH3-CH4. The Fc region of this asymmetric bispecific antibody contains one CH3 domain with residues from T-cell receptors (TCR)-α interface and another CH3 domain with residues from TCR-β interface driving heterodimerization of CH3 domains and preventing Fc mispairing [101]. Antigen-binding sites of this molecule are made up of a Fab arm specific for CD3 and a scFv arm specific for HER2. This engineered antibody exhibits the pharmacokinetics and Fc-mediated functions (*e.g.*, ADCC and CDC) like a native IgG *in vitro* and *in vivo* [100]. According to its safety and efficacy in preclinical studies, a phase I trial [ClinicalTrials.gov identifier: NCT02829372] has been conducting to evaluated tolerability and efficacy of GBR 1320 in patients with HER2-positive solid tumors. The Preliminary results showed a linear pharmacokinetics and a transient increase in inflammatory cytokine and T-cell activation within first hours of administration especially with higher dosages of GBR 1302 [102,103]. Recently, a phase I/II trial was initiated to evaluate safety and tolerability of GBR 1302 in patient with HER2-positive metastatic breast cancer [ClinicalTrials.gov identifier: NCT03983395].

2.5.7. MP0274

MP0274 is a trispecific multimer DARPin consisting of two similar domains that bind to human serum albumin to increase MP0274 half-life and two different domains which target distinct epitopes (*i.e.*, II and IV subdomains) on HER2 [104]. This DARPin interferes with both HER2- and HER3-mediated signaling which results in induction of apoptosis and significantly inhibition of the growth of HER2-positive breast cancer cells *in vitro* and *in vivo* [104]. Currently, a phase I clinical trial of MP0274 is performed to evaluate safety and tolerability and pharmacokinetics of DARPin in patients with advanced HER2-over-experssing solid tumors who had progressed after standard treatments for advanced disease [ClinicalTrials.gov identifier: NCT03084926] [105].

2.5.8. MBS301

MBS301 is a glyco-engineered (afucosylated) bispecific anti-HER2 consisting of two half antibodies (trastuzumab and pertuzumab) [106]. The two half antibodies were expressed in separate cell lines (*i.e,* dual-cell expression) and after purification, heavy chain of two antibodies were assembled *in vitro* using the knobs-into-holes technique [106]. Preclinical study confirmed that this bispecific antibody can simultaneously bind to two different binding sites of parent antibodies [106]. *In vitro* and *in vivo* anticancer model revealed that MBS301 exhibited more effective activity compared with the mix of monospecific antibodies. Furthermore, this bispecific antibody showed an improved ADCC for all CD16A genotypes than the combination of trastuzumab and pertuzumab [106]. Based on these promising preclinical results, a phase I trial study has been conducted to evaluate safety and pharmacokinetics of MBS301 for treatment of HER2-positive recurrent or metastatic malignant solid tumor [ClinicalTrials.gov identifier: NCT03842085].

2.5.9. KN026

KN026, a bispecific anti-HER2 antibody developed by Fc heterodimerization strategies (*i.e,* electrostatic steering and knob-into-hole techniques), was derived from pertuzumab and trastuzumab. Preclinical studies showed comparable or superior anticancer activities of KN026 than trastuzumab plus pertuzumab [107]. According to promising results of phase I studies, several phase II trials have been conducting to evaluated safety and efficacy of KN026 in patients with HER2-positive breast cancer and gastric cancer [ClinicalTrials.gov identifier: NCT03925974, NCT03619681 and NCT03847168].

2.6. Fusion proteins

Fusion proteins basically contain two linked moieties, *i.e.* an affinity protein such as an antibody or antibody derivative for targeting a specific tumor-related antigen and a functional protein (*e.g.*, cytokine, toxin, and enzyme) to enhance immune responses or induce direct damage to cancer cells. This section summarizes various functional proteins used for HER2-targeted therapy.

2.6.1. Cytokines

Cytokines are nature protein mediators for regulation of homeostasis, activation, proliferation, differentiation, maturation, and effector functions of immune cells [108]. To date, several types of cytokines have been identified, including interleukins (ILs), colony-stimulating factors (CSFs), interferons (IFNs), TNFs, growth factors and chemokines and most of them are involved, either directly or indirectly, in developing antitumor activity of the immune system [109]. However, application of cytokines for cancer treatment is challenging as their administration at effective dosages often causes severe side effects [110]. This issue can be potentially resolved by targeted delivery of cytokines, such as tumor targeting of cytokines *via* "immunocytokine" fusion proteins [111], enhancing the treatment efficacy with reduced cytokine dosages. Various antibody formats, including intact antibody, minibodies, scFv-Fc, diabody, and scFv, against different cancer related antigens, such as carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM), epidermal growth factor receptor (EGFR), CD20 and HER2, have been applied for the construction of immunocytokine fusion proteins with several of them being under clinical evaluation [112].

Several immunocytokines derived for HER2-targeted therapy are summarized in Table 2. In most cases, IgG3 was chosen for targeting because IgG3 is more effective for the induction of effector functions

Table 2
Some of immunocytokines for HER2-targeted therapy under preclinical evaluation.

Immunocytokine	Cytokine(s)	Targeting agent	Status	Reference
anti-HER2 IgG3–IL-2	IL-2	IgG3	Animal model	[114]
scFv-Fc–IL-2	IL-2	scFv-Fc	Animal model	[115]
IL-12–anti-HER2 IgG3	IL-12	IgG3	Animal model	[116]
anti-HER2 IgG3–GM-CSF	GM-CSF	IgG3	Animal model	[175]
IL-12–anti-HER2 IgG3–GM-CSF	IL-12/GM-CSF	IgG3	Animal model	[119]
IL-12–anti-HER2IgG3–IL-2	IL-12/IL-2	IgG3	Animal model	[119]
anti-HER2 IgG3–IFN-alpha	IFN-alpha	IgG3	Animal model	[120]
anti-HER2 scFv–TNF-alpha	TNF-alpha	scFv	*In vitro*	[122]
Anti-HER2 scFv–scTRAIL	TRAIL	scFv	Animal model	[124]

(*e.g.*, complement activation and binding to Fc gamma receptors of immune cells) [113]. The Fc region of IgG3 interacts with C1q, a component of the complement system, and activates a series of reactions leading to the elimination of pathogens and damaged cells [113]. Furthermore, IgG3 has a much longer hinge region compared with other subclasses (*i.e.*, IgG1, IgG2 and IgG4). Such molecular flexibility facilitates the derivation of fusion proteins [113]. For example, anti-HER2 IgG3–IL-2, derived by linking the carboxyl terminus of anti-HER2 IgG3 to the amino terminus of IL-2, shows a high binding capacity toward HER2-overexpressing cancer cells for maximizing the biological effect of IL-2, significantly inhibiting the growth of murine intestinal tumor cells expressing human HER2 in an *in-vivo* animal model [114].

IL-2 was also fused to the C terminus of an anti-HER2 scFv-Fc with a retained HER2 specificity and IL-2 biological activity. This immunocytokine was expressed as a bivalent form through homodimerization of Fc region. scFv-Fc–IL-2 was explored as an ADCC-mediating agent with a reasonable *in-vitro* efficacy at low ratios (*e.g.*, 20:1) of the effector immune cell (*e.g.*, NK cells, T cells) to the target cell (*e.g.*, tumor cells) and a potential improvement of the *in-vivo* therapeutic efficacy (*i.e.*, tumors growth inhibition) compared to IL-2 alone [115].

IL-12–anti-HER2 IgG3 was derived by fusing a single chain murine IL-12 with the amino terminus of anti-HER2 IgG3 *via* a flexible spacer (GGGGS)3. This immunocytokine retains the HER2-binding ability while showing the cytokine receptor specificity and heparin-binding activity similar to IL-12 alone [116]. The *mechanisms of* anti-cancer effect of IL-12–anti-HER2 IgG3 were shown to be potentially associated with a mixture of T and NK cell activities, an immune response switching from Th2 (B cell-directed) to *Th1* (T cell-directed) type, and the anti-angiogenic activity in highly complex mode [117].

Murine granulocyte macrophage-colony stimulating factor (*GM-CSF*) was also fused to carboxyl terminus of anti-HER2 IgG3 to form anti-HER2 IgG3–GM-CSF with both HER2 specificity and GM-CSF function. The *in-vivo* mouse model showed that this antibody fusion significantly reduced the growth of tumor cells expressing human HER2 and increased both Th1 and Th2 types of immune responses [118].

Bifunctional antibody fusion proteins, *i.e.*, antibodies fused with two different cytokines, have demonstrated promising antitumor activities. Two bifunctional antibody fusion proteins, IL-12–anti-HER2 IgG3–GM-CSF and IL-12–anti-HER2IgG3–IL-2, were derived to show that the combined targeted cytokines can enhance both Th-1 and Th-2 types of immune responses in immunized mice with the ECD of HER2 and effectively protect the animals against HER2-positive tumor challenge [119].

Another immunocytokine anti-HER2 IgG3–IFN-alpha was derived to improve the therapeutic index of IFN-alpha [120]. This antibody fusion protein had a longer half-life, compared to IFN-alpha alone, with a promising antitumor activity and a minimal toxicity in the HER2-positive B cell lymphoma mouse model [120].

IFN-gamma, another cytokine payload, was linked to anti-HER2 antibody moiety (4D5scFvZZ) [121]. This immunocytokine had a higher anti-proliferative activity than antibody alone or the combination of IFN-gamma and anti-HER2scFvZZ. Furthermore, the fusion protein exhibited an *in vivo* activity higher than anti-HER2 antibody in xenografted tumor models and was also effective in trastuzumab-resistant tumors [121].

TNF-alpha was also linked to the carboxyl terminus of an anti-HER2 scFv with a retained HER2 specificity and TNF-alpha biological activity [122]. However, unlike anti-HER2 scFv, the anti-HER2 scFv–TNF-alpha tended to form a trimeric structure which induced the activation of HER2 signaling and, therefore, MAPK and PI3K/Akt pathways [122]. The *in-vitro* study also showed that this trimeric antibody can inhibit apoptosis in HER2-expressing cells and facilitate repairing of the injured human colonic epithelial cells, suggesting a potential to facilitate wound healing [122].

TNF related apoptosis inducing ligand (TRAIL), a cytokine protein molecule produced and secreted by most normal tissue cells and acting as a ligand that induces apoptosis [123], has been used to derive immunocytokines for tumor targeting. A single-chain TRAIL molecule (scTRAIL), consisting of three TRAIL monomers covalently linked by the (GGGS)4 spacers, was fused to the carboxyl terminus of anti-HER2 scFv [124] to form anti-HER2 scFv–scTRAIL. This immunocytokine induced more TRAIL-mediated apoptosis *in vitro* in comparison with scTRAIL and the molecule had a longer plasma half-life with an improved antitumor activity *in vivo* [124].

Chemokine (C−C motif) ligand 5 (CCL5) or RANTES is a chemotactic cytokine which mediates the activation of leukocytes. CCL5 was linked to anti-HER2.IgG3 *via* aflexible spacer. This fusion protein simultaneously bound to HER2-overexpressing cancer cells and CCR5-expressing cells and enhanced migration of T cells and inhibited HIV infection [125].

2.6.2. Enzymes

Enzymes are biological macromolecules, *i.e.* proteins, which catalyze biological and chemical reactions. They can be effectively used for cancer treatment, especially when enzymes are selectively delivered to tumor cells using affinity proteins. Over the last two decade, genetic engineering has greatly enabled the creation of antibody-enzyme conjugates for cancer targeting. Two main class of antibody-enzyme fusion proteins have been proposed for anti-cancer targeted therapy. The first type is immunotoxins and their enzyme moiety itself has toxic effects against cancer cells (discussed in the next section). In the second type of the antibody-enzyme fusion proteins, the enzyme moiety acts as an activator for the cytotoxic prodrug [126]. This is a two-step antibody targeting approach, known as antibody-directed enzyme prodrug therapy (ADEPT), in which, a few hours after targeting cancer cells with the antibody-enzyme fusion protein, the prodrug is administrated and converted to an active cytotoxic drug selectively within the tumor (Fig. 3) [126]. However, this strategy can be compromised by immunogenicity, an immune response (*e.g.*, formation of anti-drug

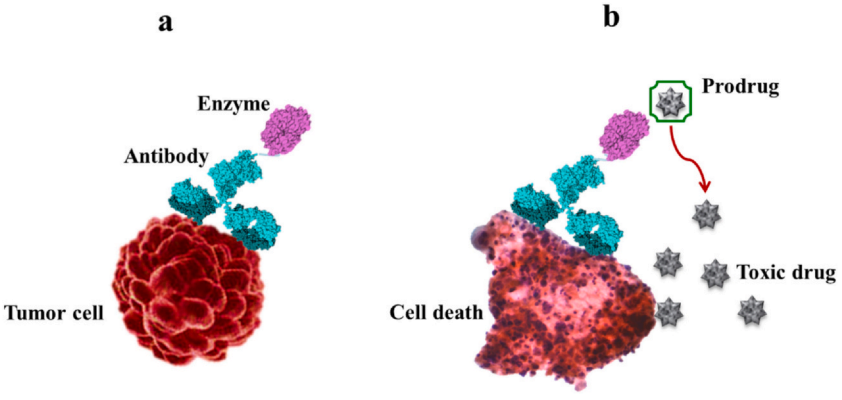

Fig. 3. Principle of antibody directed enzyme prodrug therapy (ADEPT). Antibody-enzyme conjugate which binds to antigens (*e.g.*, HER2) expressed on tumor cells is firstly administrated (a). A nontoxic prodrug is then given which converted to an active cytotoxic drug selectively within the tumor resulting in killing of cancer cells (b).

Table 3
Some of antibody directed enzyme prodrug therapy (ADEPT) strategies for HER2-targeted therapy.

Enzyme	Targeting agent	Prodrug	Status	Reference
Glutamate carboxypeptidase	IgG2 antibody	4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid	Preclinical	[127]
β-lactamase	dsFv	Cephalosporin doxorubicin	Preclinical	[128]
Purine nucleoside phosphorylase	scFv	2-fluoroadenine	*In vitro*	[130]
Deoxycytidine kinase	Affibody	Fludarabine	*In vitro*	[131]
Deoxycytidine kinase	DARPin	Fludarabine	*In vitro*	[131]

antibodies) adversely affecting the pharmacokinetics, bioavailability, and efficacy of bio-therapeutics. Furthermore, ADEPT can lead to diffusion of activated drug into tumor-surrounding cells and, therefore, non-specific killing of them, *i.e.* the bystander effect [126]. The ADEPT strategy has been applied for HER2-targeted therapy of cancers (Table 3). Various types of enzymes (*e.g.*, bacterial, fungal, plant and animal origin), prodrugs, and targeting agents (*e.g.*, IgG, Fab, scFv) have been used in preclinical studies. For example, a chemical conjugate of a rat anti-HER2 IgG2 antibody (ICR12) and a bacterial glutamate carboxypeptidase (CPG2) was derived to target HER2-overexpressing cells for activation of a prodrug derivative of a benzoic acid mustard [127]. A selective and effective cytotoxic activity of the ICR12-CPG2 conjugate, compared with conventional chemotherapy drugs, was observed in a xenografted HER2-overexpressing tumor model [127]. In another anti-HER2 ADEPT strategy, a humanized disulfide-stabilized anti-HER2 Fv (dsFv) genetically fused with a bacterial β-lactamase was used for selective activation of cephalosporin doxorubicin prodrug [128]. This treatment significantly improved targeted cytotoxic activity against HER2-overexpression tumor cells, while protecting normal cells and tissues from doxorubicin toxicity [128]. Other research group reported conjugation of a cyclodextrin-hydrolyzing enzyme (*i.e.*, maltogenic amylase (MAase)) and anti-HER2 IgG (trastuzumab) *via* dextran. This bioconjugate retained HER2 specificity of trastuzumab and enzymatic activity of MAase, significantly enhancing degradation of β-cyclodextrin inclusion complexes on HER2-overexpressing cell surface for selective release of curcumin as a model anticancer compound [129].

To decrease the potential immunogenicity during ADEPT, an antibody from human origin, *i.e.*, human scFv against HER2, was used to link with a recombinant human purine nucleoside phosphorylase for targeted activation of a non-toxic prodrug to the cytotoxic drug [130]. While this fusion protein showed a high affinity and selectivity and exhibited a targeted cytotoxicity against HER2-positive cells *in vitro*, the activation of prodrug to 2-fluoroadenine resulted in a significant bystander activity [130].

Recently, two fusion proteins were derived by respectively linking two anti-HER2 protein scaffolds, *i.e.*, affibody (10 kDa) and DARPin (16 kDa), with a human deoxycytidine kinase for activation of the nucleoside analog fludarabine [131]. Due to their small size, affibody and DARPin have a better molecular access to solid tumors, compared with other affinity proteins. This ADEPT strategy was shown to selectively enhance the anti-proliferative effect of nucleoside analogs on HER2-overexpressing tumor cells *in vitro* [131].

2.6.3. Toxins

Immunotoxins are fusion proteins consisting of a molecular targeting moiety and a toxin molecule for the expression of cytotoxicity. Immunotoxins are potent therapeutics with a lower chance of developing drug resistance than other fusion proteins, as they are functional to kill tumor cells directly, rather than through cell signaling inhibition, such that the risk of mutational escape or activation of alternative signaling pathways is minimized [132]. The toxin moiety is typically an inhibitor for protein synthesis, such as bacterial toxins (*e.g.*, *Pseudomonas* exotoxin (PE) A, *Diphtheria* toxin), plant-derived toxins (*e.g.*, ricin, gelonin), cytotoxic human proteins (*e.g.*, granzyme B, RNase), and fungal toxins (*e.g.*, ribotoxin α-sarcin). The molecular targeting moiety can be antibodies, antibody fragments/domains or ligands (*e.g.*, cytokines and hormones) directed against a variety of receptors on tumor cells. Most anti-HER2 immunotoxins are under preclinical investigations (Table 4) with two of them (*i.e.*, scFv(FRP5)–ETA and Erb-38) being in clinical trials.

scFv(FRP5)–ETA was genetically constructed by joining a scFv against HER2 (FRP5) to a shortened version of *Pseudomonas* exotoxin A (ETA) [133]. For the first application of scFv(FRP5)–ETA in cancer patients, shrinkage of cutaneous tumor lesions was observed in six of ten cases after intratumoral injection of the immunotoxin [134]. However, in another Phase I clinical study of scFv(FRP5)–ETA containing eighteen subjects with malignant solid cancers, no objective responses could be achieved after intravenous administration of the immunotoxin [135].

Erb-38 was constructed by joining anti-HER2 dsFv (e23) and PE38, a shortened form of PE with a molecular weight of 38 kDa [136]. In a Phase I study of Erb-38 consisting of six subjects with advanced carcinoma and no effective therapy, significant liver toxicity was observed in all patients and further clinical development of Erb-38 was halted [137]. The off-target activity of this immunotoxin, the non-specific binding of cytotoxic moiety to normal cells and the non-specific binding of targeting moiety to HER2 in non-targeted tissues, such as hepatocytes, may explain this serious side effect. As a result, a novel immunotoxin was recently derived by fusing a HER2-specific affibody with PE25-X7, a modified PE preserving the antitumor activity of PE38 but with a considerably lower *in-vivo* off-target toxicity and immunogenicity [138]. To improve the immunotoxin stability, an albumin-binding domain (ABD) was fused with the immunotoxin containing PE38 and anti-HER2 affibody [139]. The ABD-fused immunotoxin showed a 24.4-fold increase in plasma half-life with improved anticancer effects in an animal model, compared with the non-fused immunotoxin [139].

A chemically conjugated immunotoxin was made by using ricin toxin A-chain (RTA) as a plant-derived toxin and an anti-HER2 antibody as a targeting moiety, and the anti-HER2 IgG–RTA immunotoxin can potentially suppress the growth of HER-2 positive gastric cancer cells through the inhibitory effect on protein synthesis and induction of apoptosis [140]. Gelonin is another plant-derived toxin for use as the toxic moiety of immunotoxins. Three immunotoxins of anti-HER2 IgG (trastuzumab)–Gelonin, anti-HER2 scFv (4D5)–Gelonin and Gelonin–4D5 were produced. The construct orientation could significantly impact on biological activity of immunotoxins as Gelonin–4D5 displayed a greater cellular internalization, cytotoxicity, signal transduction inhibition, and tumor growth delay in animal model when compared with 4D5–Gelonin. While Gelonin–4D5 exhibited better tumor penetration and trastuzumab–Gelonin had longer serum half-life, they exhibited similar *in-vitro* (cytotoxic activity against cancer cells) and *in-vivo* (antitumor activity in xenografted models) anticancer effectiveness [141]. Instead of chemical or genetic fusion strategies, an enzymatic conversion using sortase A (SrtA) (a bacterial transpeptidase) was conducted for *in-vitro* ligation of the Fab of trastuzumab to Gelonin, and this Fab–Gelonin fusion protein showed a 1000-fold increase in cytotoxicity against HER2-positive cells compared to the Gelonin toxin alone [142].

To decrease the immunogenicity and toxicity of immunotoxins, toxins of human origin, such as apoptotic proteins (*e.g.*, granzyme B or

Table 4
Some of immunetoxins for HER2-targeted therapy under preclinical evaluation.

Immunotoxin	Targeting moiety	Toxic moiety	Toxin source	Additional features	Reference
HER2–PE25-X7	Affibody	PE25-X7	Pseudomonas		[138]
ABD-ZHER2–PE38	Affibody	PE38	Pseudomonas	Albumin binding domain	[139]
anti-HER2–RTA	IgG	Ricin toxin A chain (RTA)	Castor oil plant		[140]
Trastuzumab–Gelonin,Gelonin–4D5 and 4D5–Gelonin	IgG and scFv	Gelonin	Himalayan plant		[141]
Fab–Gelonin	Fab	Gelonin	Himalayan plant	sortase A (a trasnpeptidase)	[142]
hERB–hRNase	scFv	HP-RNase	Human		[144]
ERB–HP-DDADD-RNase	scFv	HP-DDADD-RNase	Human		[146]
Erb-hcAb–RNase	Compact antibody	HP-RNase	Human		[147]
FRP5-ETA$_{252-366}$–AIFA100	scFv	Apoptosis-inducing factor (AIF)	Human	Translocation domain of Pseudomonas exotoxin A	[148]
Immunocasp-6	scFv	Active caspase 6	Human	Translocation domain of Pseudomonas exotoxin A	[149]
GrB–4D5–26	scFv	Granzyme B	Human	a pH-sensitive fusogenic peptide 26	[151]
GrB–FRP5	scFv	Granzyme B	Human		[152]
trastuzumab–saporin	IgG	Saporin	Soapwort plant	Co-incubation with a photosensitizer (TPCS2a)	[156]
MH3-B1–rGelonin	scFv	Gelonin	Himalayan plant	Co-incubation with a photosensitizer (TPCS2a)	[157]
4D5–miniSOG	scFv	Mini singlet oxygen generator (miniSOG)	Arabidopsis phototropin 2		[159]

RNase), have been used as the cytotoxic moiety. Ribonuclease (RNase) is an enzyme that catalyzes the degradation of RNA and, therefore, can block intracellular protein synthesis and result in cell death [143]. RNase, practically human pancreatic RNase (HP-RNase), has been used as a cytotoxic moiety in a number of immunotoxins. For example, a fusion protein containing an anti-HER2 scFv and HP-RNase exhibited a high binding affinity and specificity to HER2-positive cancer cells and a significant *in-vivo* tumor growth inhibition [144]. A bivalent variant of this immunotoxin containing two scFv moieties, each of which was fused with one subunit of a dimeric disulfide-bond-linked RNase, showed an improved biologic activity (*e.g.*, higher specificity and binding potential, longer plasma half-life) and less sensitivity to the cytosolic RNase inhibitor, compared to the immunotoxin containing a single scFv fused with RNase [145]. Another version of HP-RNase, *i.e.* HP-DDADD-RNase in which five amino acid residues critical for the binding of RNase inhibitor were mutated, was used to reduce the immunotoxin inhibition by the cytosolic RNase inhibitor. This novel immunotoxin had more *in-vitro* anti-proliferative activity than the unaltered HP-RNase immunotoxin as the mutated variant was less neutralized by cytosolic RNase inhibitor upon cellular internalization [146]. In another study, an anti-HER2 compact antibody containing two scFv molecules linked to the Fc region of human IgG1 was fused with HP-RNase, and this fusion protein preserved both antibody-based biological activities, including inhibitory growth effects, ADCC and CDC, and RNase-based cytotoxicity toward HER2-positive tumor cells [147].

Apoptosis-inducing factor (AIF) is an apoptotic protein that can be used to form immunotoxins. A truncated form of AIF was fused with the scFv(FRP5)–PE domain II and showed a high toxicity toward cancer cells *in vitro* [148]. In another study, scFv(eb23)–PE domain II fusion protein was further joined to caspase-6, an apoptotic enzyme. Note that PE domain II, a nontoxic translocation domain, was used in this fusion protein for translocation of caspase-6 into tumor cells, and this immune-conjugate exhibited a significant inhibition of tumor growth and metastasis in a xenografted HER2-overexpressing osteosarcoma tumor model [149].

Granzyme B (GrB) is a serine protease secreted by cytotoxic effector cells (*e.g.*, cytotoxic T-lymphocytes (CTL) and NK cells) to kill its targeted cells *via* the induction of apoptosis [150]. To generate a fully human immunotoxin, an anti-HER2 scFv (4D5) was linked to GrB and a pH-sensitive fusogenic peptide 26 was added at the C terminus of the construct to facilitate endosomal scape and provide a higher concentration of the immunotoxin in the cytoplasm of target cells. The targeting fusion GrB–4D5–26 can inhibit the proliferation and survival of cells resistant to lapatinib or trastuzumab [151]. Another group reported the construction of a fusion protein containing GrB and scFv (FRP5) and observed that the immunotoxin selectively killed cancer cells in the presence of chloroquine, an endosomolytic reagent causing lysis or disruption of endosome [152].

Many macromolecules including immunotoxins can be taken up *via* receptor-mediated endocytosis to enter the cell as receptor-macromolecule complexes in endocytic vesicles entrapped in early endosomes with a neutral pH [153]. Unless the receptor-macromolecule complexes have specific destinations, such as organelles other than endosome, they will be transported to late endosomes with an acidic pH [153]. Degradation of immunotoxins by acidic pH or hydrolases in endocytic vesicles was suggested as the major mechanism for the resistance of tumor cells to the targeted treatment [154]. Therefore, the release of immunotoxin from endocytic vesicles to cytosol can be applied as an escape mechanism from endosomal degradation. Photochemical internalization (PCI) is a recent approach in which a photosensitizer, which is a light-sensitive molecule generating singlet oxygen upon its photoactivation, and a therapeutic macromolecule of interest are simultaneously administrated. Then, light is used to activate the photosensitizer, resulting in the disruption of endosome and release of endocytosed macromolecules [155]. In an *in-vitro* study, the cytotoxic

effect of PCI of a chemically conjugated immunotoxin containing trastuzumab and saporin was evaluated and it was found that the treatment sequence (*i.e.* light exposure after the administration of the immunotoxin) is important for a successful PCI-therapy [156]. In another study, PCI-mediated delivery of anti-HER2 scFv–Gelonin immunotoxin was shown to be effective for killing both low and high HER2-expressing breast cancer cell lines [157]. In addition, PCI-therapy improved the *in-vivo* effectiveness (*e.i,* tumor growth inhibition) of anti-HER2 scFv–Gelonin for targeted treatment of ovarian cancer cells xenografted athymic mice and PCI-mediated delivery of the immunotoxin was well tolerated in animal model [158].

A novel immunophototoxin was genetically constructed by linking a highly phototoxic small protein, mini Singlet Oxygen Generator (miniSOG) which generates singlet oxygen upon an exposure to blue light, to an anti-HER2 scFv (4D5). The singlet oxygen can act as a primary cytotoxic agent by rapidly interacting with cellular components. Upon the photo-activation, scFv (4D5)–miniSOG showed a selective cytotoxicity against HER2-overexpressing breast cancer cell lines [159].

2.7. Conjugates

In addition to therapeutic proteins mentioned above, the affinity (*i.e.*, antibody) moiety can be fused with other types of therapeutic agents, such as drugs, chemical toxins, or radioisotopes, to form therapeutic conjugates for targeted disease treatment.

2.7.1. Drugs

ADCs are potentially attractive for cancer therapy as they possess both the efficacy of small-molecule therapeutics and the targeting ability of an antibody [160]. Recently, several ADCs against HER2 have been developed and some of them are under clinical and preclinical trials.

ARX788 contains an anti-HER2 IgG site-specifically conjugated with Amberstatin269, a potent cytotoxic microtubule inhibitor which suppresses microtubule dynamics and inhibits mitosis leading to cell cycle arrest and apoptosis, *via* a non-natural amino acid linker [161]. Because of its site-specific conjugation, the biophysical properties and drug stability of this anti-HER2 ADC has been improved, resulting in a higher cancer treating efficacy camped with T-DM1 (*i.e.*, due to its neglectable drug deconjugation, a wider range of ADC doses can be administrated that has antitumor activities and yet prevents undue toxicities) [161]. A clinical trial of phase I/II for multiple-dose escalation study of ARX788 has been initiated for treating patients with HER2-overexpressing metastatic cancers [ClinicalTrials.gov identifier: NCT02576548].

RC 48 (disitamab vedotin) is composed of a humanized anti-HER2 antibody (*e.i,* hertuzumab) with higher affinity to HER2 compared with trastuzumab, conjugated *via* a cleavable spacer to monomethyl auristatin E, a tubulin inhibitor agent which prevents cell division and induces apoptosis [162]. Preclinical data showed that this ADC had higher antitumor activity than T-DM1 in mice xenografted with breast cancer cells resistant to trastuzumab and lapatinib. Preliminary results of three ongoing phase I trials [ClinicalTrials.gov identifiers: NCT02881138, NCT02881190 and NCT03052634] indicate a reasonable efficacy (a response rate of 37–57%) and a manageable safety profile (only a few patients experienced serious adverse events) of RC 48 in subjects with HER2-positive breast carcinoma [163–165]. Recently, three phase II clinical trials [ClinicalTrials.gov identifiers: NCT03507166, NCT03809013 and NCT03556345] have been conducted to evaluate the efficacy and safety of RC 48 in patients with advanced HER-expressing urothelial and gastric cancer in China. In April 2020, based on promising results of these studies, FDA approves Investigational New Drug (IND) application to initiate RC 48 US-based Phase II trial in HER2-positive metastatic or unresectable urothelial cancer.

SYD985 is another novel HER2-targeting ADC that consists of the prodrug form of duocarmycin, a bacterial cytotoxic small molecule, and trastuzumab. *In-vitro* and *in-vivo* studies found that SYD985 has a cancer-treating potency comparable to a previously approved ADC, T-DM [166]. Based on the promising results of the preclinical studies in mice and primates and the unique pharmacodynamic (*i.e.*, having antitumor activity against both HER2- positive and HER2-low cancer cells as a result of the bystander effect) and pharmacokinetic (*i.e.*, great stability and neglectable deconjugation) properties of SYD985 [167], a phase I clinical study was conducted for treating advanced or metastatic solid tumors [ClinicalTrials.gov identifier: NCT02277717]. The preliminary results suggested reasonable efficacy and tolerability of SYD985 in both HER2-positive and HER2-negative advanced breast cancer subjects [168]. Accordingly, FDA has granted fast track designation to this agent in January 2018. Currently, SYD985 is entered a phase III trial to evaluate its efficacy and safety compared with physicians' choice in patients with advanced HER2-overxpressing breast cancer who had failed treatment with T-DM1 [ClinicalTrials.gov identifier: NCT03262935].

2.7.2. Radioisotopes

Immuno-radio conjugates are composed of an antibody-targeting moiety and a cytotoxic radioisotope for selective internal radiotherapy. Various therapeutic radioisotopes, including α-particle emitters (*e.g.*, ^{212}Pb, ^{227}Th), β-particle emitters (*e.g.*, ^{131}I), and Auger-electron emitters (*e.g.*, ^{111}In), are used to label targeting agents [169]. Several radiolabeled intact anti-HER2 antibodies, including pertuzumab and trastuzumab, were evaluated in preclinical or clinical trials.

A conjugate composed of a bifunctional chelating agent, 1,4,7,10-Tetra-(2-Carbamoyl Methyl)-Cyclododecane (TCMC), and trastuzumab was labeled with an α-emitting ^{212}Pb. This α-emitter antibody conjugate, ^{212}Pb-TCMC-trastuzumab, exhibited a high-level radiation selectively toward cancerous cells. In addition to preclinical studies, a phase I clinical trial using an intraperitoneal ^{212}Pb-TCMC-trastuzumab was conducted to evaluate the safety, pharmacokinetics, immunogenicity, and anticancer effectiveness of this agent in patients with HER2-positive malignancy, and a promising drug tolerability was observed [170,171].

^{227}Th-trastuzumab was another immuno-radio conjugate composed of an anti-HER2 IgG covalently linked to an α-particle emitter *via* a 3,2-hydroxypyridino-based chelator. In preclinical investigation, ^{227}Th-trastuzumab was even more efficacious than trastuzumab and T-DM1 [172]. Currently, a phase I trial is recruiting participants to evaluate the safety, pharmacokinetics, tolerability and anticancer efficacy of BAY2701439 (*i.e.*, ^{227}Th-trastuzumab) in patients with advanced HER2-positive cancer [ClinicalTrials.gov identifier: NCT04147819].

A β-emitter single domain antibody conjugate, ^{131}I-SGMIB-anti-HER2 nanobody, was developed for treatment of HER2-positive cancer. SGMIB (N-succinimidyl 4-guanidinomethyl 3-iodobenzoate) is a linker enhancing tumor internalization and trapping of radioiodine, resulting in improved efficacy and reduced renal toxicity of this immuno-radio conjugate [173]. Based on promising preclinical data, a phase I study was conducted to evaluate a low radioactive dose of ^{131}I-SGMIB-anti-HER2 nanobody in healthy volunteers and HER2-positive breast cancer patients. It was reported that the immuno-radio conjugate tended to accumulate in tumor lesions of breast cancer patients. Safety and pharmacokinetic results revealed that ^{131}I-SGMIB-anti-HER2 nanobody exhibited a rapid bio-distribution and kidney clearance with no significant adverse effects [174].

3. Conclusion and future perspectives

Due to its key roles in initiation and progression of tumors, HER2 has been identified as an effective target for cancer prevention, diagnosis and treatment. One of the most interesting anti-HER2 therapeutics is an affinity protein which selectively recognizes and specifically binds to a defined target. Different types of affinity proteins including

antibodies, antibody fragments and other protein scaffolds (*e.g.*, affibody) have been developed for HER2-targeted therapy. As discussed in this review, some of these proteins have been approved (*i.e.*, trastuzumab, pertuzumab, T-DM1, and Fam-trastuzumab deruxtecan-nxki) and many others are under preclinical and clinical evaluations for treatment of breast cancer and other HER2-overexpressing cancers. Advances in genetic engineering and biotechnological technology have led to the generation of novel fusion and conjugate molecules, bispecific/trispecific antibodies and antibodies with optimized Fc that demonstrated promising activity in preclinical and clinical studies.

Fc engineering techniques such as glyco-optimization and amino acid mutations have been recently used to increase or reduce the effector functions (*e.g.*, ADCC, CDC) and to modulate the half-life of therapeutic antibodies. Fc engineered ani-HER2 antibodies (*e.g.*, margetuximab, trastuzumab-GEX) have more anticancer efficacy, less adverse effect and even being effective in trastuzumab-refractory or HER2-low subjects. Using advanced technologies for site specific conjugation of anti-HER2 led to the development of novel ADCs agents (*e.g.*, DS-8201a and SYD985) which unlike T-DM1 are active in both HER2- positive and HER2-low cancer patients thanks to their bystander effect. However, more clinical studies are still needed to confirm their efficacy and safety. On the other hand, construction of multifunctional and multispecific agents (*e.g.*, immunontoxin, immunocytokine, bispecific antibody) has recently attracted much attention. A multispecific molecule triggers different targets at the same time and as a result exhibits superior efficacy (*e.g.*, synergistic effect, simultaneous targeting of different cell types or biochemical pathways, overcoming tumor resistance) and safety (*e.g.*, less off-target and adverse events) when compared with a combination therapy consisting of monospecific molecules for each target. However, production of traditional bispecific antibodies usually leads to lower yield and purity (*e.g.*, due to misparing of their chains and generation of homodimers (*i.e.*, monospecific antibodies)) and these multispecific proteins generally exhibited lower solubility and stability compared with their parent monospecific ones. Novel technologies such as asymmetric platform of bispecific antibodies (*e.g.*, BEAT®) or multispecific non-immunogloboline scaffolds (*e.g.*, DARPin) can overcome these limitations. Furthermore, smaller formats of antibody (*e.g.*, scFv, nanobody) or alternative scaffolds (*e.g.*, DARPin, anticalin®, affibody) are easier and cheaper to produce and modify compared with intact antibodies. They also have shorter half-lives and better tumor penetrations making them more suitable for diagnostic application (*e.g.*, imaging), although they are not proper candidates for therapeutic aims due to their fast clearance (*i.e.*, elimination from plasma) and need to a multiple-dosage regimen to maintain the plasma drug level. Different strategies such as fusion with ABD or conjugation with PEG have been applied to enhance the half-life of small-size anti-HER2 affinity proteins. Using advanced labeling techniques, many radioconjugates have also been generated that could be applied for cancer therapy as well as diagnostic imaging and prediction of patient's response to anti-HER2 treatments. Intrinsically or acquired drug resistance is still a major challenge for anti-HER treatments of cancer. Various and complicated pathways play role in the refractory to anti-HER2 therapy and the exact mechanisms of drug resistance are still need be explored. Therefore, more comprehensive and well-designed translational and clinical experiments must be performed to demonstrate the contributing factors. Biomarkers, genetic polymorphisms and imaging analysis play a critical role in appropriate patient selection reducing the chance of drug resistance. Furthermore, targeting simultaneously HER2 and other biomarkers (*e.g.*, HER3, VEGF, CXCR4) taking part in cancer pathogenesis will help us to overcome drug resistance.

Approved anti-HER2 affinity proteins are mostly used in breast cancer patients. However, there are many ongoing clinical trials evaluating safety and efficacy of former and novel anti-HER2 affinity proteins in other HER2-expressing cancers (*e.g.*, gastric). Development of novel HER2-targeted therapies and combinations with enhanced selective anticancer activity and minimal side effects will be further explored in the future which will increase the clinical effectiveness in patients with HER2-overexpressing cancers. However, there is still an increasing demand for inexpensive HER2-targeted agents through the introduction of novel development, production and manufacturing processes.

Declaration of Competing Interest

The authors report no conflict of interest.

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