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Nanotechnology in the development of small and large molecule tyrosine kinase inhibitors and immunotherapy for the treatment of HER2-positive breast cancer

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Abstract

The HER2 receptor tyrosine kinase is a member of the epidermal growth factor receptor family which includes EGFR, HER3 and HER4. They are known to play critical roles in both normal development and cancer. A subset of breast cancers is associated with the HER2 gene, which is amplified and/or overexpressed in 20–25% of invasive breast cancers and is correlated with tumor resistance to chemotherapy, Metastatic Breast Cancer (MBC) and poor patient survival. The advent of receptor tyrosine kinase inhibitors has improved the prognosis of HER2-postive breast cancers; however, HER2+MBC invariably progresses (acquired resistance or de novo resistance). The monoclonal antibody-based drugs (large molecule TKIs) target the extracellular binding domain of HER2; while the small molecule TKIs act intracellularly to inhibit proliferation and survival signals. We reviewed the modes of action of the TKIs with a view to showing which of the TKIs could be combined in nanoparticles to benefit from the power of nanotechnology (reduced toxicity, improved solubility of hydrophobic drugs, long circulation half-lives, circumventing efflux pumps and preventing capture by the reticuloendothelial system (mononuclear phagocyte system). Nanotherapeutics also mediate the synchronization of the pharmacokinetics and biodistribution of multiple drugs incorporated in the nanoparticles. Novel TKIs that are currently under investigation with or without nanoparticle delivery are mentioned, and nano-based strategies to improve their delivery are suggested. Immunotherapies currently in clinical practice, clinical trials or at the preclinical stage are discussed. However, immunotherapy only works well in relatively small subsets of patients. Combining nanomedicine with immunotherapy can boost therapeutic outcomes, by turning “cold” non-immunoresponsive tumors and metastases into “hot” immunoresponsive lesions.

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INTRODUCTION

The Human Epidermal Growth Factor Receptor 2 (HER2) is a type I transmembrane glycoprotein that plays a pivotal role in activating the downstream signaling cascades that control cell proliferation, survival, and apoptosis in HER2-positive breast cancer [1]. The ErbB receptor family of Receptor Tyrosine Kinases (RTKs) comprises four distinct receptors: the EGFR (also known as ErbB1/HER1), ErbB2 (neu, HER2), ErbB3 (HER3) and ErbB4 (HER4) [2,3]. HER2 can dimerize with itself or with other EGFR family members, thus activating downstream signal transduction pathways of tyrosine phosphorylation, and eventually resulting in the regulation of various cellular functions [2,3]. The ErbB receptors are triggered by several peptide ligands, comprising TGF-α, EGF, amphiregulin (for EGFR), and neuregulin (for HER3 and HER4) [2].

Ligand binding triggers receptor dimerization, activation of the kinase domains, auto-phosphorylation, and initiation of down-stream signaling. However, HER3 contains no kinase domain (which can be activated) and HER2 has no known ligand (that binds its receptors). Therefore, HER2 and HER3 depend on mutual dimerization with each other and other ERBB receptors to initiate their cellular effects [2]. HER2 is moderately expressed in normal adult tissues; however, an average of 20%–30% of human breast cancers overexpress HER2 receptors by amplification of the HER2/neu gene, which is a marker of aggressive cancer with an unfavorable prognosis [4] that correlates with tumorigenesis and metastasis [5–7]. HER2 is also overexpressed in approximately 20% of gastroesophageal junction and gastric cancers with an equally poor prognosis [3,8].

Several bioactive agents that target HER2 positive breast cancer have been approved by the FDA and are currently being used in the clinic (Figure1). Lapatinib was the first small molecule TKI approved by the FDA in 2007 [9] followed by neratinib in 2017 [10], pyrotinib in 2018 (only in China) [11] and tucatinib in 2020 based primarily on the outcome of the HER2CLIMB clinical trial [12]. The first drug developed in the class of large molecule TKIs is the monoclonal antibody, Trastuzumab (TRZ), which was approved in 1998. The approval of Trastuzumab (TRZ) was followed by the development of Pertuzumab (PTZ) in 2012 and Phesgo ® (Fixed Dose Combination of TRZ and PTZ) in 2020 as an option to improve efficacy and patient compliance based on results from the landmark CLEOPATRA study [13]. A recent entrant is margetuximab that was approved in December 2020 for use in anti-HER2+ experienced patients with metastatic HER2-positive breast cancer [14]. In a bid to increase efficacy, patient compliance and reduce the adverse effects of the partner cytotoxic agents, anti-HER2 monoclonal antibodies have been conjugated with cytotoxic partners. A popular example is adotrastuzumab (T-DM1: TRZ conjugated to emtansine) which was approved in 2013 and is currently the second line in several guidelines [15].

Despite the clinical successes recorded with these bioactive agents due to their targeted design, resistance to monoclonal antibodies [16,17] remains a challenge in addition to the toxicity of cytotoxic partners which invariably affects their efficacy, safety, and compliance in the treatment of HER2-positive breast cancer. It is on the backdrop of these problems that the design of novel drug delivery systems has been proposed: encapsulation of cytotoxic drugs in nanoparticles which are then decorated by HER2 specific monoclonal antibodies for targeting two or more drugs simultaneously to neoplastic tissues.

Nanotherapeutics is an important and fast emerging field that is focused on the design of nanocarriers from biocompatible and biodegradable components (proteins, polymers, peptides, RNA/DNA aptamers, lipids, liposomes, and inorganic materials) for the delivery of bioactive agents in the treatment and diagnosis of HER2 positive breast cancer and other applications [18]. To achieve the goal of eliminating death and suffering from cancer, the USA National Cancer Institute has embraced the power of nanotechnology to radically change the procedure for diagnosis, imaging and treating of cancer. The integration of nanotechnology with advances in cancer research can be done using nanoparticles which are capable of specific delivery of large amounts of single or multiple therapeutic agents as well as imaging agents embedded in the core per targeting biorecognition event compared to simple immune-targeted drugs. Both targeting (spatial/distribution control) and controlled release (temporal control) of therapeutic agents can be achieved [19–21]. Nanoparticles for biomedical applications have evolved from the first-generation, suitable for liver targeting, through the second generation of stealth nanoparticles for long blood circulation and passive targeting to the third generation with molecular recognition [22,23] and fourth generation (theranostics): that incorporates diagnostic/reporter agents in the same nanodevice [24].

Site specific nano-based drug delivery has the advantages of passive tumor targeting stemming from high permeability and retention effect (EPR), active targeting via the coupling of receptor specific ligand to carriers, lower toxicity [25,26] of encapsulated drug and intracellular endocytosis uptake which mediates evasion of mechanisms of multidrug efflux pump systems including p-glycoprotein [27]. In addition, nanotherapeutic carriers can be potentially functionalized to enhance drug-carrying capacity for delivery of multiple therapeutics to cancer cells simultaneously [18,28]. The controlled drug release attribute of nanoparticle systems can normalize and synchronize the pharmacokinetics, biodistribution, bioavailability and stability of multiple drugs that possess very different physicochemical properties that would have independently produced contrasting pharmacological behaviors if administered separately [28]. The use of nanocarriers enables the formulation of FDCs (Fixed Dose Combinations) with the capacity for ratiometric dosing, providing the ability to tailor the relative ratios of each agent based on their respective pharmacological disposition [29]. Further, therapeutic nanoparticles can improve the solubility of anticancer drugs when encapsulated in the hydrophobic core while surface stealth property of incorporated water-soluble polymers, like PEG, prevents opsonization and capture by the Reticuloendothelial System (RES). Dose reduction with better quality of life during chemotherapy would improve patient compliance [30]. The advantages of nanotherapeutics have led to the approval of several non-targeted nanodrugs including Doxil®, Thermodox®, Myocet® Genexol® and Opaxio™. These products have the advantages of lower toxicity (prevention of immediate release of cytotoxic components) and longer circulation time either by the

impact of surface pegylation and/or controlled release based on the ratio of specific nanomaterials used. Nevertheless, these products still lack the desired specificity required for targeted delivery at the site of neoplasia, stability in blood circulation as well as issues with the emergence of novel toxicities such as Palmar Plantar Erythrodysesthesia (PPE) and exanthema, oral mucositis, and hematological toxicities [31,32]. It is hoped that the conjugation of targeting ligands will overcome these challenges and lead to better therapeutic outcome.

It is important to differentiate between Antibody Drug Conjugates (ADCs) in which a partner cytotoxic agent is covalently linked to the antibody as seen in T-DM1 and Antibody-Conjugated Nanoparticles (ACNPs) which are designed to exploit the benefits of both antibody conjugation (such as site-specificity) and nanotechnology [33,34].

While ADCs can be targeted via the use of ligands that bind endogenous targets, they are unable to deliver drugs in a controlled manner; neither can they maintain the chemical structure of conjugated partners, avoid irregular metabolism, nor diminish toxicity when compared to ACNPs [35]. Moreover, ACNPs present the extra benefit of increasing endocytosis which translates to higher tumor cell permeability and accumulation, and stability against multi-resistant efflux pumps. Here we review the development of nanoparticle based small and large molecule Tyrosine Kinase Inhibitors (TKI) that are currently in clinical practice, in clinical trials or at the preclinical stage. Mention will be made of novel TKIs that are currently in development with or without nanoparticle delivery, and nano-based strategies to improve their delivery will be proposed.

LARGE MOLECULE TYROSINE KINASE INHIBITORS (TKI)

Trastuzumab

Trastuzumab is the premier humanized murine monoclonal antibody developed for the treatment of HER2 positive breast cancer. The efficacy of trastuzumab has been demonstrated in both neoadjuvant and adjuvant treatment of Metastatic Breast Cancer (MBC) as shown in several studies: CLEOPATRA [16,36]; TRYPHAEN [37]; KATHERINE [38]; EMILIA [39], THR3ESA [40] and MARIANNE [41]. Trastuzumab is recommended by both St. Gallen and National Comprehensive Cancer Network (NCCN) guidelines for use as monotherapy after adjuvant treatment [15]. Current guidelines recommend dual-blockade and the most preferred first line of treatment is a combination of trastuzumab + pertuzumab while T-DM1 and lapatinib are considered as second and third line options respectively [2,3,15].

Mechanism of action

Trastuzumab acts by binding to the Extracellular Domain (ECD) IV of the HER2 receptor with high specificity and avidity to block downstream signaling, attracting immune cells and inducing internalization of the antibody-receptor complex leading to inhibition of cell growth and proliferations [17,18,42–44]. These effects are elicited in various ways and are manifested via intracellular and extracellular mechanisms [42,44–46]. First, by binding the domain IV on the ECD of the HER2 receptor, trastuzumab is recognized by the

Fcγ receptors expressed on the surfaces of cells of the innate immune system, including APCs (Antigen-Presenting Cells), NK cells (Natural Killer), and effector immune cells that readily eliminate TRZ tagged tumor cells via Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) [44]. Second, TRZ inhibits HER2 shedding. In some breast cancer-derived cell lines, the ECD of HER2 is proteolytically discarded from the cell surface [47,48] and the residual truncated receptor ('p95 HER2' or 't95 HER2') [49], relieved of autoinhibition by the receptor's ECD), engages in constitutive tyrosine kinase activity [50,51] to promote cellular signal transduction. By binding to HER2 receptor, TRZ blocks this proteolytic shedding, either through a steric or allosteric mechanism, thus preventing or downregulating HER2 kinase activity [50,52]. Finally, TRZ stimulates endocytosis and degradation of HER2 domain hence preventing HER2 homodimerization [44,52] and HER2/HER3 heterodimerization that are required for downstream signal transduction. This is achieved through TRZ mediated recruitment of c-CBL, an E3 ubiquitin-protein ligase involved in cell signaling and protein ubiquitination [53,54]. The impact of these mechanisms leads to the inhibition of HER2 downstream pathways including MAPK and PI3K/AKT/mTOR thus downgrading cell growth and proliferation [17]. Other confirmed mechanisms associated with the therapeutic effect of TRZ include inhibition of angiogenesis, induction of cell-cycle arrest and apoptosis [55] and interference with DNA repair [56–57].

Trastuzumab (TRZ) Nanoparticle Formulations

There is currently no nanoparticle formulation of TRZ in the clinic [18,58] neither is any in ongoing clinical trials as revealed by ClinicalTrials.org. In the only trial involving nanoparticles (NCT01730833), TRZ was not conjugated to paclitaxel albumin-stabilized nano-formulation. That notwithstanding, TRZ conjugated nanoparticles have been studied extensively in numerous preclinical trials involving cell cultures and animal models to explore several novel strategies to increase the precision of tumor targeting, tissue accumulation and cellular uptake of various nano-encapsulated cytotoxic and theranostic agents.

In one study [27], TRZ was electrostatically linked to a cationic lipoplex nanoparticle (Figure 2) formulated from PEG, PEI, and lipids (LPP), with a payload of curcumin and doxorubicin (LPPC) based on the ability of LPP component to strongly adsorb various biologically functional proteins on its surface (presence of NH_2) which are difficult to displace by proteins in vivo [59]. In addition to its adsorptive effect, LPP demonstrated capacity to protect the structure of encapsulated drugs against oxidation as well as provide a strong cytotoxic effect against drug-resistant tumor cells [60,61]. The effect of this ACNP against HER2 positive SK-BR-3 (HER2 positive) breast cancer cell line in xenograft mouse model of HER2+ breast cancer showed that 50% of the mice treated with LPPC/doxorubicin/TRZ ACNPs were tumor free during 66 days of observation translating to 4x the lifespan of the group treated with PBS (Phosphate Buffered Saline). PEI and PLA (Poly Lactic Acid)-based NPs conjugated TRZ have been reported in a strategy [62] to deliver dasatinib for the purpose of rescuing TRZ resistance in HER2+ BT474 and resistant BT474-HR cell lines. Dasatinib is a multikinase inhibitor of SRC, a tyrosine kinase transducer whose alteration has been implicated in TRZ resistance [63,64]. The use of high

molecular weight (MW 25,000) PEI may limit the translation of this strategy since it is well documented that HMW PEI is toxic in *vivo* due to its proton sponge effect, though this can be limited by using low MW PEI, PEGylation or by blocking the excess NH_2 groups. The last option may increase the particle size of the LPPC. It should be noted that although covalent conjugation involves several steps and may be time consuming; however, it still provides a more stable conjugate compared to conjugates formed from electrostatic interaction (used in the cited work) which may be hampered by the heterogeneous nature (proteins, salts etc.) of the *in vivo* environment leading to desorption of the conjugated ligand thus diminishing its specificity.

One of the challenges of targeted delivery using monoclonal antibodies is the need to strike the sweet spot between the total number of moieties conjugated to the surface of a nanocarrier and the minimization of immunological reactions due to recognition and rapid clearance by macrophages and other scavenger cells. An attempt to quantify the absolute number of conjugated mABs has been demonstrated in a liposome Nano-carrier (NC) system [65]. In this report, docetaxel was incorporated in a liposome system terminated with Mal-PEG and later mixed with an already thiolated trastuzumab for surface conjugation to terminal Mal-PEG in various ratios of (508:1, 1.127, and 1.16:1) to determine the ratio with the best affinity and minimal immunological challenge. Exposure of these immunoliposomes to a MDA-MB-453 spheroid showed a significant difference in cell viability between the liposomes, free docetaxel and control. However, there was no significant difference in cell viability among the immonoliposomes. This result bolsters the opinion that a high mAB surface density may not necessarily translate to site specific avidity probably due to certain factors including steric hinderance. Leakage of docetaxel and DiD fluorescent molecules from the hydrophilic phase affected proper quantification of the conjugated mABs. Nevertheless, this formulation has shown superiority when indexed against free docetaxel, free TRZ and TDM-1 [66].

The concept of *in situ* click chemistry with trans-cyclooctene and Tetrazine (TCO/Tt cycloaddition) has been explored in a two-component, two-step, pre-targeting drug delivery system integrated with image guidance to overcome the challenge of resistance and non-specific toxicity associated with ADCs [5]. In this study, TRZ was conjugated to TCO and a fluorescent agent to form $[Tz(TCO)_6(CF\text{-}680)_2]$ while paclitaxel was conjugated to albumin and another fluorescent agent to form ALB(Px)2.6(PEG4-Tt) (CF-750). The click treated, fluorescent labelled trastuzumab $[Tz(TCO)_{2.6}\ (CF\text{-}680)_2]$ was injected i.p into mice with orthotopically placed tumor xenografts and given enough time to bind to HER2+ expressing tumor cells. The click treated $ALB(Px)_{2.6}$(PEG4-Tt) (CF-750) was injected 12 hours later to activate click reaction with the already bound click treated TRZ as shown in Figure 3. This strategy harnesses the close proximity of overexpressed HER2 receptors on cancer cells and multiply derivatized pre-targeting and delivery components, leading to multiple cross-linking reactions which induces self-assembly of cell membrane-bound nanoclusters *in situ* which are effectively internalized by clathrinmediated endocytosis [5,67]. The result showed a superior cellular uptake of this formulation as monitored *via* imaging compared to control. The extent of tumor regression in the click treated group was substantially better than the untreated and mock treated controls.

The synchronized delivery of TRZ and neratinib with PAMAM (polyamidoamines) dendrimers has been described [68]. Neratinib was loaded into the core of a G4 PAMAM dendrimer before conjugating the NP surface with FITC (Fluorescence isothiocyanate) for imaging. Thereafter, a heterocross-linker, [maleimide-poly(ethylene) Glycol-N-hydroxysuccimide (NHS-PEG-MAL)] was covalently linked to the NPs followed by bioconjugation with an already thiolated TRZ. The resulting nanocarrier was shown to have superior cytotoxic effect on SK-BR-3 (HER2+) cells when compared to free neratinib most likely due to the targeting ability and anti-HER2 property of TRZ. The synthesis of the NPs had several steps which could interfere with the loading efficiency and drug release as acknowledged by the authors considering the 19.24% loading efficiency achieved and a 10% decrease in drug release. In terms of clinical translation, dendrimers present a challenging prospect because of their non-specific toxicity and tendency to accumulate in the liver due to their high cationic density. Besides, the LMW dendrimers are rapidly cleared *in vivo* and there is a poor control over the release profile of encapsulated drugs [69].

In another strategy [70], the combination of ultrasound with TRZ conjugated nanobubbles (PTX-NBs-HER) serving as a contrast agent and drug carrier has been explored for image-guided drug delivery and precise targeted therapy at the tumor site. The nanoparticles were prepared by the double emulsion method (W/O/W) from PLGA in which paclitaxel was encapsulated and made into nanobubbles by passage of perfluoropropane gas before linkage to TRZ. On administration, the nanobubbles were delivered to the tumor site through the tropism of TRZ and the encapsulated paclitaxel released by the application of exogenous ultrasound stimulus which triggered controlled release of the drug as well as cavitation of the cell membrane to promote enhanced permeability and retention (Figure 4). The mechanism relies on ADCC and enhanced endocytosis promoted by TRZ and the cytotoxic effects of paclitaxel which accelerate tubulin polymerization and inhibition of depolymerization, resulting in the formation of stable non-functional microtubule bundles, thus extinguishing mitosis and cell proliferation [70]. PLGA tagged TRZ has also been used to safely deliver epirubicin to MDA-MB-453 and BT-20 HER2+ cell lines [71] with success. The dose dependent side effect of epirubicin including cardiotoxicity [72] has limited its clinical application. Therefore, the translation of a PLGA-TRZ-epirubicin targeted nanocarrier will be a daunting proposition due to possible additive cardiotoxic effect when epirubicin is combined with TRZ.

The role of other exogenous ligands in the efficient and targeted delivery of gene therapy to the nucleus of tumor cells has been reported [73]. In this publication, nanoparticles were formed from a 10:1 ratio of NHS-PEG_{3500}-Mal and 25kDa PEI which was linked to either antiHER2 nanobody (RR4) or TRZ in the presence of Traut's reagent. Terminal PEI groups were subsequently linked to a genetic material-p15BID (tbid), a proapoptotic gene and its promoter pMUC1. The results showed that the highest expression of tBid gene and consequently the least amount of cell viability in HER2-positive cells (SK-BR-3 and BT-474) was observed in the group treated with TRZ modified nanoparticles. This result was largely due to the efficient delivery and expression of the killer gene in these cells. Low levels of specificity and targeting was observed with the low/non-HER2 expressed cell line (MCF-10A) thus signifying the safety potential of this design towards healthy cells. It is worth noting that this nanobody (RR4) led to good gene delivery and expression, hence

presenting an opportunity for further exploration in targeted tumor delivery in preclinical and clinical studies.

Inorganic materials have been used for the delivery of multiple payloads to solid tumors. Examples include gold, SPIONs (Supra Paramagnetic Iron Oxide Nanoparticles), carbon nanotubes, quantum dots, gold, and silica, to mention a few [74]. They are particularly attractive because of the ease of synthesis, the large surface area to volume ratio, capacity for derivatization that allows the electrostatic or covalent linkage with several surface conjugates, and the ease of scale up. In a recent study [75], radiotoxic alpha-emitter Ac radionuclide was incorporated into SPIONs produced via the co-precipitation method to form 225Ac@Fe3O4 nanoparticles. The NPs were linked to the surface of Fe_3O_4 nanoparticles via a 3-phosphonopropionic acid (CEPA) linker to form Ac@Fe3O4-CEPA nanoparticles that were conjugated to TRZ at pH 8.0 in a borate buffer in the presence of NHS/EDC. *In vitro* incubation of SKOV-3 cells to the plain 225Ac showed a surviving fraction of 67.7 ± 4.3%, 16.9 ± 6.6% for 225 Ac@Fe3O4-CEPA nanoparticles and 7.9 ± 1.5% for 225Ac@Fe3O4-CEPA-trastuzumab radio-bioconjugate. These outcomes clearly indicate that the TRZ conjugate is more toxic to SKOV-3 cells than other unvectorized NPs. However, *in vivo* biodistribution of these conjugates showed a considerable uptake by cells of the RES in the liver and spleen of SKOV-3 tumor-bearing SCID mice due to high hydrodynamic size of the NPs. The poor accumulation of these NPs at the tumor site due to sequestration by the RES [76,77], the propensity of radionuclides to decay into toxic metabolites that can cause genotoxicity, poor water solubility [78,79] and poor biodegradation [74,80] are some of the drawbacks of inorganic nanoparticles that make their translation to the clinic a daunting task. Nevertheless, inorganic materials in form of ultrasmall silica NPs linked to TRZ have been used for dual drug delivery and theranostic purposes [1].

PEG coating on NPs provides stealth property that aids evasion of the RES thus improving the circulation time of nano-constructs. However, it has been observed that when layers of targeting moieties are conjugated directly to PEG molecules on the surfaces of NPs, the stealth property is diminished [81]. In a strategy to improve the hydrophilicity of conjugated systems, a study [81] explored the concept of linking another PEG molecule to an already conjugated TRZ. Nanoparticles were formed by the nanoprecipitation method from diblock copolymers, PLGA-b-PEG-N3 and PLGA-b-Mpeg before clicking to TRZ via a 2kDa NHS-PEG-alkyne linker. Another short linker of NHS-PEG-methoxy with a MW of 333 Da was used to modify the already conjugated TRZ at a molar ratio of 200:1. Cellular uptake studies of single and double modified TRZ in SK-BR-3, MCF-7, and MDA-MB-231 showed that SK-BR-3 had the highest cellular uptake for all the conjugated NPs. This result showed that contrary to expectation, additional PEG did not alter the affinity of the modified TRZ for HER2 receptors. Exposure to RAW 264.7 cells revealed that the doubly modified PEG NPs with 10% and 50% azide heads showed a 70% and 82% reduction, respectively, in uptake and elimination by RAW 264.7 cells compared to their single modified counterparts.

PERTUZUMAB

Pertuzumab is a recombinant humanized monoclonal antibody that binds to a distinct epitope, precisely the subdomain II of the extracellular domain of HER2 [82]. Pertuzumab is composed of two heavy chains with 449 amino acid residues and two light chains with 214 residues in an immunoglobulin G1 kappa framework [83]. It is the second most popular monoclonal antibody used for HER2+breast cancer treatment.

Mechanism of Action

Pertuzumab inhibits ligand-dependent HER2 signaling thus preventing ligand-activated HER2/HER3 or HER2/HER1 heterodimerization, a potential escape mechanism from the inhibitory effect of trastuzumab [82,84]. Pertuzumab inactivates multiple downstream signaling networks [85] and triggers ADCC [86] like TRZ but differs in being effective in cases of normal HER2 and high HER1 (EGFR) levels, or breast cancers with characteristically low HER2 expression [43].

The efficacy of pertuzumab has been demonstrated in several studies. In the landmark CLEOPATRA study, pertuzumab in combination with docetaxel and trastuzumab led to a 40% overall response rate (ORR), significantly improving Progression-Free Survival (PFS) and Overall Survival (OS) with multiple complete and partial responses [13], hence approval of this combination as a first-line treatment in HER2-positive MBC [85]. In particular, the median overall survival was extended by more than a year and reached an excess of 4.5 years [2]. Furthermore, pertuzumab was the first drug to be approved with an endpoint of Pathological Complete Response (PCR) in the neoadjuvant setting [39]. In the NeoSphere study [87,88], neoadjuvant combination of pertuzumab + doxetacel + trastuzumab achieved superior pCR compared to pertuzumab + docetaxel, and trastuzumab + docetaxel. Pertuzumab monotherapy is well tolerated and shows no increase in cardiotoxic effect when added to trastuzumab or anthracycline free cytotoxic regimen [87,89]. The pharmacokinetic profile of pertuzumab has been shown to be stable in both monotherapy and combination therapy (trastuzumab/docetaxel) with no currently reported Drug-Drug Interaction (DDI), indicating that a Fixed Dose Combination (FDC) is feasible with pertuzumab [13,82,85,89].

Pertuzumab Nano-Formulations

There are no nano-formulations of pertuzumab in the clinic nor in ongoing clinical trials as assessed from Clinical trials.gov. In a recently published paper from our laboratory [90], a technique leading to nanoparticles fabrication by *in situ* dispersion polymerization (free of surfactants), involving the use of biodegradable macromonomer, a pH sensitive crosslinker, a redox initiator system and a hydrophilic macromonomer to provide stealth attributes was described. Specifically, PEG-monomethacrylate 2000 was conjugated to pertuzumab *via* carbodiimide (CDI) reaction followed by hydrazide derivatization. Assessment of cell viability in SKBR-3 cell line (HER2-positive) showed a dose dependent reduction in cell viability comparable to unmodified pertuzumab, though the free mAB had superior impact at lower concentration. However, at high concentration (30μm), PEG-pertuzumab had a

drastic effect on cell viability, considerably decreasing cell population as it became more active.

Margetuximab

Margetuximab is a chimeric IgG1 monoclonal antibody that targets domain IV of the extracellular loop of HER2 receptor like trastuzumab [14,45]. The difference lies in the optimization of the Fcγ region via the substitution of five amino acids which increases the binding affinity for CD16A polymorphs and a decreased affinity for FcγRIIB (CD16B) that inhibits the activity of trastuzumab. Such reduced affinity frees the Fc region of margetuximab to bind more tightly to effector cells (macrophages and NK cells) leading to increased ADCC [91]. Margetuximab preserves binding to the high affinity activating FcγRI (CD64) [92] hence conserving the antiproliferative properties of trastuzumab.

Mechanism of Action

Margetuximab-cmkb binds to domain IV of the extracellular loop of HER2 receptor. It inhibits tumor proliferation, reduces shedding of HER2 extracellular domain (a resistant mechanism) and binds tightly to effector cells to mediate strong ADCC [14,93].

Margetuximab (Margenza) received approval by the FDA in December 2019 as part of a chemotherapy regimen for treatment of anti-HER2 experienced adult patients with metastatic HER2-positive breast cancer [94]. This approval was based on the successes recorded in clinical trials including the phase I study, **MGAH22** [95] which revealed margetuximab as a promising single agent in the treatment of experienced patients with HER2-positive solid tumors. In this study, the objective response rate (ORR) was 17% and 3 out of 4 responders remained on treatment for 39 months-54 months. Unlike trastuzumab, no cardiotoxicity was observed. Interim analysis in the **SOPHIA** trial [96] showed that margetuximab improved primary PFS(RR=24%) and ORR (24% vs 22%) over trastuzumab at different time points. The frequency of infusion-related reactions in the first cycle was higher with margetuximab compared to trastuzumab (35 [13.3%] vs 9 [3.4%]); however, they were both comparable with respect to other side effects. Other planned trials of margetuximab include the **MAHOGANY** trial (NCT04082364) [97] and **MARGOT** study (NCT04425018). There are currently no nano-formulations of margetuximab either in preclinical or clinical trials.

ANTIBODY DRUG CONJUGATES (ADCS)

Trastuzumab emtansine (TDM1)

Ado-trastuzumab emtansine (Kadcyla™, Genentech/Roche) was the first developed HER2 ADC synthesized by the conjugation of TRZ to emtansine *via* a non-cleavable thioether linker-MCC (maleimidomethyl cyclohexane-1-carboxylate) [17]. TDM1 was designed to harness the cytotoxic effect of emtansine on tubulin polymerization and the targeted delivery/antibody mediated cell cytotoxicity of TRZ [58,98]. Emtansine is an inhibitor of microtubules and is about a thousand times more potent than doxorubicin and paclitaxel [99,100]. When administered alone, emtansine, like other cytotoxic agents, harms both healthy and cancerous cells; however, conjugation to TRZ prevents this nonspecific effect

since TRZ ensures active targeting of only HER2+ neoplastic tissues thus enhancing the overall clinical outcome for patients [101].

Mechanism of Action of TDM1

Upon administration, T-DM1 selectively binds exquisitely to subdomain IV of the HER2 receptor on the surface of antigen-positive cells via the natural tropism of TRZ [17]. The interaction of TRZ with HER2 receptor leads to endocytosis [102], degradation of endosomes on fusion with lysosomes [103] and liberation of MCC-DM1 which navigates through the nuclear membrane to interfere with the polymerization of tubulin [104]. Specifically, inhibition of tubulin assembly into a functional mitotic spindle prevents the important nexus between kinetochores on chromatids and the spindles, hence inducing cell-cycle arrest in metaphase culminating in ultimate mitotic disaster [105].

Trastuzumab related mechanism: TDM1 retains the mechanism of action of TRZ which include inhibition of the homo and heterodimerization of HER2, HER1, HER3, and HER4 receptors, activation of ADCC, inhibition of domain shedding [54], angiogenesis and DNA repair [27].

The efficacy of TDM1 has been demonstrated in several studies [39–41,106]. In the EMILIA study, subjects with HER2-positive metastatic breast cancer previously treated with trastuzumab and a taxane showed improvements in median overall survival (OS) and PFS in the T-DM1-treated group compared to subjects treated with lapatinib and capecitabine in the control group [39]. This result was also replicated in the THR3SA study [40]. In the MARIANNE trial [41], TDM1 either alone or in combination with pertuzumab were not superior to TRZ/taxane; however, they were better tolerated than TRZ/taxane. Like EMILIA and THR3SA, TDM1 in the MARRIANE study equally had a prolonged PFS compared to TRZ/taxane. In the KRISTINE study [106], TDM1/pertuzumab was inferior to TRZ/pertuzumab/docetaxel/ carboplatin; however, 44% of subjects achieved pCR without exposure to a retinue of potentially toxic systemic chemotherapy, clearly indicating that a subgroup of patients with particularly high and homogeneous HER2 expression, may not necessarily need neoadjuvant chemotherapy. This trend was equally observed in subjects in the Phase 2 PREDIX trial [107]. In the KATHERINE study, TDM1 administered to subjects with HER2-positive early breast cancer with residual invasive disease showed a lower risk of recurrence when compared to adjuvant trastuzumab, though with less tolerable outcome [38].

Despite the efficacy of TDM1 in HER2+breast cancer treatment, several resistance mechanisms have been reported. These include reduced expression of HER2 receptors [17], upregulation of alternative RTKs [108], increased expression of efflux transporters (MRP1,2&4, MDR1, P glycoprotein and BCRP) [109–112], impaired lysosmal release of lysine MCC-DM1(active DM1 metabolite) and enhanced recycling of HER2–T-DM1 complexes which is associated with reduced DM1 intracellular release [110,113]. Literature is replete with comprehensive reviews of TRZ and TDM1 resistance mechanisms. It should be recalled that unlike other members of the ErBb family, HER2 has no natural ligand, and this is thought to be instrumental to its impaired lysosomal trafficking [113,114]. As a result, HER2 is largely recycled back to the plasma membrane after spontaneous

endocytosis [17]. Consequently, only a small fraction of T-DM1 is channeled to lysosomes for degradation and release within the cytoplasm [115] thus contributing to poor treatment outcome. Therefore, it is imperative to deploy other strategies including nano-delivery systems since they are not susceptible to extrusion by efflux pumps to overcome the dual challenge of resistance and poor delivery of TDM1 [116].

TDM1 Nano-Formulations

A search of the literature and clinical trials.gov revealed no nanoparticle formulation of TDM1 either in clinical practice or ongoing trials. At the preclinical stage, the most recent study by Rong et. al. [117] that conjugated TRZ to a three-arm star-shaped block copolymer of D-α-tocopheryl polyethylene glycol 1000 succinate-poly (D, L-lactide) (PLA-TPGS) in which TDM1 was incorporated, was withdrawn on 1/11/2021 due to an error in the representation of an image that could result in misleading interpretations.

Novel ADCs

In an attempt to overcome the HER2 receptor recycling partly responsible for T-DM1 resistance, newer ADCs are designed with a cleavable drug linker that mediates bystander killing effect. This is the passive extension of the cytotoxic effect of free toxins via diffusion from target HER2-positive cancer cells to neighboring cancer cells that cannot be targeted due to limited or complete absence of receptor expression. This effect is beneficial in the overall efficacy of ADCs that target HER2+ receptor, considering the inherent heterogeneity of HER2-positive breast neoplasia [45]. However, this design is also the Achille's heel of most ADCs as evidence has shown that they are intrinsically toxic and can produce severe side effects due to their long circulatory half-life and toxicity in healthy tissues [118]. This concern has led to the withdrawal of a couple of ADCs in development as discussed in this section. There are no nano-based formulations of these products besides XMT1522 as most are still in early development.

Preclinical ADCs

A major impediment in the treatment of HER2+ breast cancer is the inherent intratumoral heterogeneity which has been observed in 16%−36% of patients with HER2+ tumors that is associated with aggressive tumor cell proliferation, high relapse rates, and poor survival [119–122]. An attempt to accelerate the treatment of highly heterogenous tumors with a homogenous ADC coupled to two distinct payloads has been published recently [123]. In this work, MMAE (monomethyl auristatin E) or MMAF (Monomethyl Auristatin F) were linked to Paraaminobenzyloxycarbonyl (PABC), glutamic acid-valine-citrulline cleavable linker, a PEG spacer and, terminal DBCO (Dibenzylcycolooctyne) and TCO (Transcyclooctyne), respectively. In a separate reaction, an anti-HER2 derivatized N297A trastuzumab was coupled to a "tri-arm linkers" with azide and methyl tetrazine heads via the transpeptidase action of Microbial Transglutamase (MTGase) at the glutamine side chain. The functionalized TRZ was then conjugated to the previously synthesized payloads via cycloaddition of azide/DBCO and Methyltetrazine/TCO pair to produce a branched double loaded ADC. There is inherent synergy in this ADC as MMAE has a pan-cytotoxic effect on a broad range of breast cancer cells but is susceptible to extrusion by efflux pumps; however, MMAF does not possess a bystander effect due to poor diffusivity but is resistant

to the action of efflux pumps. Assessment of the cytotoxic effect of different ratios of MMAE and MMAF in cell lines with highly expressed HER2 (KPL-4, and SK-BR-3), refractory HER2+(JIMT-1), and HER2 negative (MDA-MB-231) cancer cell lines showed a considerable cytotoxic effect only in the HER2+ cell lines- with EC_{50} values of 0.017 nM-0.029nM in KPL-4 cells, 0.024nM-0.045nM in JIMT-1 cells, and 0.18nM - 0.34nM in SK-BR-3 cells. *In vivo* evaluation in orthotopically placed mice model of heterogeneity (MDA-MB 231+JIMT-1) demonstrated high efficacy in tumor regression while maintaining a comparable pharmacokinetic and safety profile. The safety concerns of ADCs are well known, and a good safety profile makes this dual construct a promising option in the treatment of HER2+ breast cancer. Further safety measures can be built in by encapsulating the payloads in nanoparticles before clicking to trastuzumab tri-arm linkers. This will ensure a gradual *in vivo* release as well as help MMAE overcome the problem of extrusion by efflux pumps.

Li and co-workers [115] designed a biparatopic antibody consisting of a Fab domain made by the fusion of a single chain variable fragment (scfv) derived from TRZ to the heavy chain of 39S Fv designed de novo via hybridoma technology. The biparatopic monoclonal antibody was then conjugated to a maleimidocaproyl functionalized tubulysin AZ13599185, an inhibitor of microtubule polymerization. The efficacy of this construct was tested in a panel of cell lines with varying (high, SKBR-3; low, RT-112; limited, T47D and resistant, JIMT-1) HER2 expression and the results showed extensive cytotoxicity in cells with limited HER2 expression as observed in TNBC (Triple Negative Breast Cancer). Some mechanisms advanced for this outstanding effect involves clustering of the ADC at the cell surface, efficient endocytosis, inhibition of receptor recycling to the cell surface and bystander effect. It has been documented that avidity at receptor site on the cell surface accelerates antibody internalization, inhibition of recycling, and lysosomal degradation [124–126]. This biparatopic ADC can be deployed differently by conjugating it to an appropriate nanoparticle that incorporates tubulysine and another small molecule that targets metastatic HER2 positive tumors through less permeable barriers like the BBB. The triple synergy will harness the existing advantages of tubulysine and the monoclonal antibody in addition to the impact of another cytotoxic agent, and the controlled delivery, efflux pump evasion and enhanced endocytic effect of nanoparticles.

Trastuzumab Deruxtecan (DS-8201/T-DXd) manufactured by Daiichi Sankyo, is a humanized monoclonal IgG1 anti-HER2 ADC of trastuzumab, a self-immolative maleimide linker, and a topoisomerase-I payload-deruxtecan [127,128]. T-DXd shares a common mechanism of tumor delivery with TDM-1, however, preclinical studies demonstrated broader anti-tumor activity that extended to low HER2 expressing tumors [45] compared to T-DM1. The broader effect of TDXd has been attributed to a better handling of T-DM-1 resistance mechanisms due to differences in linker and payload, stability against extrusion by efflux transporters and bystander effect [110,112,127]. T-DXd efficacy has been demonstrated in phase 1 trials [129,130]. In the landmark DESTINY Breast 03 trial [131], among the 261 patients in the T-DXd arm, the 12-month PFS rate was 75.8% while the 12-month OS rate was 94.1%. T-DXd is currently approved as a second line [132] for anti–HER2 experienced patients with unresectable, metastatic HER2-positive breast cancer

[133]. Aside T-DXd, other emerging strategies for targeting HER2+ BC with ADCs are summarized in Table 1.

Novel ADCs in clinical trials

Bispecific Antibodies—Bispecific antibodies (BsAbs) are designed to harness the benefits of dual inhibition of different targets or epitopes, either in the same or different receptors. For instance, BsAbs can inhibit signal transduction in two or more RTK signaling pathways by deactivating either the RTKs or their ligand. Several BsAbs are currently being studied in patients with advanced HER2-positive disease [45].

Bispecific Nanodelivery Approach—It was thought that the inhibition of more than one epitope on the HER2+ receptor will improve inhibition of signal transduction compared to mono-inhibition. This is the basis for the development of Phesgo®, a fixed dose combination of pertuzumab and TRZ that inhibits domain II and IV of the HER2+ receptor, respectively. To harness this strategy while incorporating a cytotoxic agent in a nano-delivery system, a study [134] designed a bispecific anti- mPEG/anti- HER2 antibody (BsAbs) conjugated to a docetaxel (DTX)-loaded methoxy-Pegylated lecithin-stabilized micellar drug delivery system (LsbMDDs) for targeting HER2+ cells (Figure 5). DTX was encapsulated in an amphiphilic polymer DSPE-PEG2K (1,2-Distearoyl-sn-glycero-3-phosphoethanol-amine-N[methoxy(polyethyle- neglycol), in a 1:5 ratio to form lecithin-stabilized NCs (LsbMDDs).

The anti-mPEG/anti-HER2 BsAbs delivery system comprised a Fab fragment linked to the methoxy ends of the mPEG on LsbMDDs surface, and a free scFv moiety that targets HER2+ receptors on tumor cells. On exposure to HER2+ SKBR-3 and MDC-7 cell lines, BsAbs-LsbMDDs demonstrated better release and biodistribution that ensured sustained delivery over a long period compared to controls. BsAbs-LsbMDDs also demonstrated a higher cellular uptake by SK-BR-3 and HER2+/MCF-7 cell lines compared to HER2−/MCF-7 cell lines (due to poor targeting) while there was 1.3–2.3 folds higher biodistribution and a 5 folds-10 folds higher retention of DTX at the site of action when indexed against comparators in *in vivo* evaluation. Other bispecific mABs are summarized in Table 1.

SMALL MOLECULE TYROSINE KINASE INHIBITORS (SMTKIS)

In general, SMTKIs are oral non-peptide anilinoquinazolone compounds [144] that are homologous to Adenosine Triphosphate (ATP) [145] and compete reversibly or irreversibly at the cytoplasmic catalytic kinase domain, preventing tyrosine phosphorylation, and consequently, inhibiting the activation and transduction of downstream signal pathways [146,147]. Since cancer cell survival and proliferation are aided by HER2 and its homodimerization or heterodimerization with HER1, HER3, or HER4 [148], inhibition of EGFR function disrupts EGFR-HER2 crosstalk resulting in a variety of therapeutic effects [145]. The smaller size of SMTKIs compared to mABs makes them useful in treating Metastatic Brain Cancer (MBC) because of the inherent ability to cross the BBB more effectively [45].

Lapatinib

Lapatinib is a dual inhibitor of EGFR, HER2 and p-glycoprotein [149] that was approved in 2013 in combination with TRZ as a chemotherapy-free regimen for patients with HR-negative and HER2-positive advanced BC [150]. The efficacy of lapatinib alone or in combination with other therapy in MBC (including brain metastases) [151] has been proven in many studies [152,153].

Mechanism of action

Lapatinib reversibly binds to the cytosolic ATP domain of EGFR and HER2 receptors to inhibit tumor cell proliferation primarily through the PI3K/Akt and MAPK pathways [154]. Inhibition of tyrosine kinase activity results in reduced phosphorylation of RAF, ERK, Akt, and PLC1 proteins[155–157]. Lapatinib also inhibits Insulin-Like Growth Factor I (IGF-I) signaling in trastuzumab-sensitive and trastuzumab-resistant HER2 overexpressing cells [158]. This is important because crosstalk between the IGF-I receptor and HER2 in trastuzumab-resistant cells increases HER2 receptor phosphorylation [151,159] leading to tumor cell proliferation. Further, lapatinib increases fragmentation of poly ADP-ribose polymerase (PARP), a protein implicated in programmed cell death, and downregulated survivin expression [160,161]. In HER2-positive breast cancer cells, lapatinib regulates numerous microRNAs (miRNAs), which play crucial roles in anti-tumor activity [162–164]. In this context, lapatinib treatment increases the expression of miR575 and miR1225–5 resulting in the downregulation of the oncogenic enzyme phospholipase C PLCXD1 (phosphatidylinositol-specific phospholipase-C-X-domain-containing-1), an miR-575 and miR1225–5p target gene [164].

Lapatinib nanoparticle formulations

According to ClinicalTrials.org, there is presently no nanoparticle formulation of lapatinib in clinical use or ongoing trials. Regardless, lapatinib conjugated nanoparticles have been extensively examined in several preclinical experiments involving cell cultures and animal models. The concept of pH-responsive nano-delivery has been explored in a study [165] with lapatinib. In this evaluation, lapatinib was encapsulated in NPs formulated from mesoporous silica materials, decorated with PEG (ImIL-PEGylated@MCM-41) before sealing of the porous surface with covalently linked imidazolium-based ionic seal to achieve controlled release (Figure 6). The presence of N-methyl imidazole ionic liquid seal on the porous surface of the NPS in a neutral medium prevents premature release [166]; however, in an acidic/hypoxic environment, the seal breaks down to release the loaded drug due to hydrolysis of imidazolium ionic liquid [167]. The result of this study showed that when Lap@ImIL-PEG@MCM-41 was exposed to SK-BR-3 cell lines and control (HEK-293), there was less cell viability in SK-BR-3 cells compared to HEK. This was primarily attributed to lapatinib's affinity for HER2 receptors, excellent lapatinib entrapment efficiency and extended controlled release property of the nanocarrier. In general, the surface density of silanol groups interacts readily with phospholipids on the red blood cell membranes, leading to hemolysis-a drawback for mesoporous silica nanoparticles [168]. However, in this study, hemolysis was mitigated via the incorporation of PEG in the nanocarrier to reduce interaction between RBCs and the mesoporous silica layer.

Other novel nanodelivery strategies with lapatinib have been used for the treatment of other types of Breast cancer; they could be applied to treating HER2+ BC. In one study [169], an attempt was made to design pH-responsive nano-delivery systems without conjugation to ligands that are prone to opsonization and clearance by the RES [170]. In this study, lapatinib was loaded into pH-responsive, charge switchable, polycarbonatedoxorubicin conjugate micelles for synergistic breast cancer treatment. This design takes advantage of the knowledge that anionic NPs have long circulation time in blood; however, they can be easily repelled by the negatively charged cell membrane. One way to circumvent this challenge is switching of polarity to cationic charge at the target site to induce local fluidization causing cell membranes to lose their rigidity thus allowing in rapid internalization and penetration across multiple cell layers [152,153,171].

The rich glutathione (GSH) level in tumor cells relative to normal cells has been explored in intracellular drug release triggered by disulfide redox sensitivity [172–174]. In a study that exploited this strategy nanoparticles encapsulating several hydrophobic anticancer drugs including Gefitinib (GEF), Lapatinib (LAP), Olaparib (OLA) were synthesized via the nanoprecipitation method with polydisulfide amide (L-Cystine Dimethyl Ester Dihydrochloride (Cyst-8E)) and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine polyethylene glycol 3000 (DSPE-PEG_{3000}) to form SMA-NPs (Small Molecule Anticancer-Nanoparticles) [175]. Evaluation of the *in vivo* effect of LAP-NPs showed a strong and efficient suppression of tumor volume compared to free LAP and control NPs. Particularly, the tumor inhibition rate for LAP-NP was 62.5% compared to 42.29% for free LAP which reflects the extent of tumor volume regression. This study relies solely on EPR and would benefit from active targeting if conjugated to HER2 specific ligands.

Neratinib

Neratinib is an oral pan-HER TKI that has demonstrated promising anti-tumor results in trastuzumab pretreated individuals based on the phase III ExteNET trial [176]. In combination with capecitabine, neratinib decreased CNS lesions by 49% in the TBCRC 022 study [177]. The incidence of CNS tumor recurrence and time to CNS metastases has been shown to be significantly lower (p=0.002), (HR 0.45 95% CI: 0.26–0.78, P=0.004) [177], respectively, with neratinib. It has demonstrated clinical activity with a response rate of 69% in patients with prior taxane, trastuzumab, and lapatinib therapy [16].

Mechanism of action

Neratinib binds covalently and irreversibly to a unique and shared cysteine residue in the ATP-binding pocket of the EGFR family of receptors [178]. In addition, neratinib binds to the cysteine residues Cys-773 and Cys-805 in the HER1 and HER2 proteins, respectively [179] thus inhibiting PI3K/Akt and MAPK downstream signaling induced by HER2 receptor activation [45,180,181] resulting in the stagnation of the G1-S phase transition, increase in p27 (cyclin dependent Kinase inhibitor), and decrease in Phosphorylated Retinoblastoma Protein (PRB) and cyclin D1 [148,182] that are involved in cell proliferation. Neratinib has been shown to reverse membrane-bound ATP transporter-mediated multidrug resistance in cancer cell lines [183] thus providing an alternative method for improving chemotherapy response in HER2-positive breast cancer. However, concurrent suppression of wild-type

EGFR leads to common toxicity of diarrhea associated with neratinib. This has limited neratinib's clinical application [184].

Following a search of the literature and clinical trials.gov, there is no nanoparticle formulation of neratinib in clinical practice or current trials. A study that synchronized delivery of TRZ and neratinib has been discussed earlier in this review [68].

Tucatinib

Tucatinib is an orally active reversible inhibitor of the MAPK and P13K/AKT signaling pathway via inhibition of the cytosolic tyrosine kinase domain of HER2 and HER3 receptors [185]. Tucatinib is highly selective for HER2 [186] has no effect on EGFR, and thus fewer side effects (rashes and diarrhea) compared to other small molecule TKIs [148].

Mechanism of action

In vitro and *in vivo* studies revealed tucatinib as a competitive and reversible ligand that binds the ATP pocket of the cytosolic domain of the HER2 receptor, blocking HER2 phosphorylation and thereby suppressing signal transduction downstream the MAPK and PI3K/AKT pathways [185]. Its selectivity confers a safety and tolerability profile that favors combination therapy with various therapeutic agents, including monoclonal antibodies and ADCs (Antibody-Drug Conjugates) [187]. Besides, tucatinib demonstrates a more significant potential to cross the BloodBrain Barrier (BBB) when compared to monoclonal antibodies; hence its application in therapies aimed at treating and preventing brain metastases, which occurs in 30%−50% of patients with advanced HER2-positive breast cancer [188].

A search of the literature and clinical trials.gov did not turn up any published article on nano-formulations of tucatinib either in clinical practice or clinical trials. In addition, a search of the literature for tucatinib conjugated nano-formulations revealed no article in the preclinical setting.

Pyrotinib

Pyrotinib is a new oral small molecule TKI that inhibits cell proliferation via the PI3K/AKT/mTOR and MEK/MAPK pathways and acts against numerous HER family members (HER1, HER2, and HER4) [189,190]. It specifically binds to ATP binding sites in the intracellular kinase sections of HER1, HER2, and HER4, effectively blocking the downstream signaling pathways initiated on the tumor cell membrane by homodimers or heterodimers of the HER family [191]. In two clinical trials [192,193] pyrotinib in combination with other HER2 therapies, was found to have a greater progression-free survival rate than placebo. However, based on our literature search, nanoparticle formulations of pyrotinib are yet to be reported.

Afatinib

Afatinib is an orally active, second-generation anilinoquinazoline derivative that inhibit members of the ErbB receptor family by binding irreversibly to an intracellular tyrosine kinase domain [194,195]. The irreversible binding properties of afatinib may help impede

mutant cell lines, such as EGFR L858R/T790M mutations, which are typically resistant to erlotinib and gefitinib [196]. The FDA approved afatinib only for treatment of metastatic Non-Small Cell Lung Cancer despite its effect on HER2 receptor. This is largely due to toxicities observed during Phase III [197,198] trials in spite of apparent efficacy in Phase I and II trials. Nano-formulation strategies are currently underway to reduce toxicity and investigations are focused on evaluating efficacy and safety in lung cancer [199,200] with no mention of such investigation in HER2+BC.

Most studies reported on nanotechnology formulations are largely reliant on the EPR mechanism, especially among studies involving small molecule TKIs [165,201,202]. It is our strong believe that TKIs can benefit from strategies that incorporate active targeting of tumor cells. Hence there is ample opportunity for various research groups to exploit the numerous targeted delivery techniques discussed in this review for enhancing the efficacy of TKIs in combination with other antineoplastic agents.

THE ROLE OF NANOTECHNOLOGY IN IMMUNOTHERAPY IN HER2-POSITIVE BREAST CANCER

A new generation of treatment modality that stimulates the host immune system to selectively kill cancer cells with relatively fewer side effects on healthy tissues has been dubbed cancer immunotherapy; it also promotes systemic immune surveillance that can eliminate both primary and metastatic tumors. Compared to chemotherapy, surgical resection and radiotherapy, the anti-tumor immune cells activated by immunotherapeutic strategies can even kill Circulating Tumor Cells (CTCs) and thus inhibit the formation of metastatic foci. Further, immunotherapy can establish a long-term immune memory to prevent tumor recurrence [203]. These immunotherapeutic agents are exemplified by cytokines, checkpoint blockers, anti-cancer vaccines and Chimeric Antigen Receptor T-Cells (CAR-T).

Checkpoint Blockers for Cytotoxic T-lymphocyte antigen 4(CTLA4)

Advances in cancer therapy in the past few years have included the development of medications that modulate immune checkpoint proteins. Cytotoxic T-lymphocyte antigen 4 (CTLA4) and programmed death 1 (PD1) are two coinhibitory receptors that are expressed on activated T cells to which therapeutic blocking antibodies have reached routine clinical use [204]. To prevent the immune system from causing harm to the host when reacting to a foreign antigen, humans have evolved immune checkpoint proteins and mechanisms to quickly halt an immune response. However, in the case of cancer, malignant cells have developed many mechanisms to evade the human immune system, including the ability to limit immune responses through such immune checkpoints. Monoclonal antibodies that target CTLA4, such as ipilimumab (approved by the FDA for treatment of unrespectable or metastatic melanoma), have demonstrated efficacy in cancer treatment. With CTLA4 blocked, activated T cells proliferate and achieve a persistent state of activation, which enables the targeting of otherwise poorly immunogenic tumor antigens on cancer cells [205]. Tremelimumab works through a similar mechanism as ipilimumab to block CTLA4.

Checkpoint Blockers for Programmed death-1 (PD-1) and PD-L1

Programmed death 1 (PD1) is a protein on the surface of T cells of the immune system and programmed death ligand 1 (PD-L1) and ligand 2 (PD-L2) are on the tumor cells and antigen presenting cells. The interaction of PD-1 and its ligand (PD-L1) in the Tumor Microenvironment (TME) provides an immune escape for tumor cells by turning off cytotoxic T cells (when bound to a ligand, PD1 lowers the threshold for apoptosis, induces energy via blunted T-cell receptor signaling, and generally leads to T-cell depletion). Thus, blocking this interaction subjects the tumor cells to attack from cytotoxic T cells. In certain tumor cells, upregulation of PD-L1 expression has been seen, which leads to increased inhibition of T-cell activity in favor of tumor cell survival. A monoclonal antibody against PD1 can block this pathway (that is a PD1–PDL1 interaction) and can result in the upregulation of immune response and inhibition of tumor growth. Suppressing these immune checkpoints results in immune-mediated antitumor activity in mouse models and clinical trials. Thus recent advances in the understanding of tumor biology and immune checkpoint molecules (PD-1/PD-L1) have provided novel therapeutic strategies using Immune Checkpoint Blockade (ICB). Pembrolizumab and nivolumab were approved by the FDA for the treatment of patients already treated with ipilimumab for unresectable or metastatic melanoma and disease progression [207]. Both antibodies inhibit the interaction between PD1 and its ligands and increase the immune response against cancer cells and are efficacious against non-small cell lung cancer, renal cell cancer, bladder cancer and Hodgkin lymphoma [204]. The advent of anti–programmed death ligand 1 (PD-L1) monoclonal antibodies have transformed the paradigm of treatment in a variety of solid tumors, including the treatment of Non-Small Cell Lung Cancer (NSCLC). The U.S. Food and Drug Administration (FDA) granted approval to atezolizumab and durvalumab in March of 2019 and 2020, respectively, for use in combination with chemotherapy for first-line treatment of patients with small cell lung cancer. Further, combined CTLA4 and PD1 blockade has been explored in preclinical models and clinical trials in metastatic melanoma [204].

Chimeric antigen receptor T cell (CAR-T)

Chimeric antigen receptor T cell (CAR-T) therapy is an innovative form of immunotherapy in which autogenous T cells are genetically modified to express chimeric receptors encoding antigen-specific fragments and various co-stimulating molecules. Unlike physiological T cells with common T cell receptors, these modified CAR-T cells are transported to and recognized by cancer cells in ways unrelated to Human Leukocyte Antigens (HLA). At present, CAR-T therapy has performed well in the treatment of hematologic malignancies, but challenges remain in solid tumors such as breast cancer. Many targets are under study for CAR-T cell therapy for BCs such as anti-HER2 CAR-T cells (NCT02547961) [207]. T cells against HER2 can be successfully expanded ex vivo in mice models and have shown evidence of antitumor activity [208]. One patient with HER2+MBC was treated with autologous HER2369–377 T cells that were cocultured with HLA2-peptide-loaded DCs in a pilot trial [209]. There was evidence of tumor cell disappearance in the bone marrow but no penetration of T cells into the tumor. HER2 CAR T cells containing CD28 costimulatory domain were administered to the Central Nervous System (CNS) of mice and showed regression of HER2+ MBC in the CNS [210].

Breast Cancer

PD-Ll is a therapeutic target in breast cancer, and inhibition of PD1/ PD-Ll signaling has shown extremely promising signs of activity in breast cancer [211,212]. Triple-negative Breast Cancer (TNBC) has been found to have high immunogenicity among BC subtypes. Renewed attention and research directions have been attracted toward the immunotherapy for breast cancer, especially on TNBC [213]. The FDA granted an accelerated approval for the immunotherapy drug (atezolizumab) in combination with chemotherapy (nab-paclitaxel: Abraxane) for the treatment of women with advanced TNBC (for tumors that are positive for a protein called PD-L1). It is known that HER2-expressing breast cancer cells use the PD-1/PD-L1 cells checkpoint axis to evade cytotoxicity by immune system. Further, PD-L1, a ligand for PD-1, is constitutively expressed in a subset of HER2+ breast cancer patients. Higher PD-L1 expression on HER2+ breast cancer has been shown to have a significant positive correlation with a higher tumor grade and tumor-infiltrating lymphocytes [42]. Preclinical studies in immune-competent mice showed that PD-1 and CTLA-4 inhibition improves the immune-mediated effects of HER2-targeted therapies through synergistic activation of CD8+ T cells. These data provide a rationale for the clinical development of immune checkpoint inhibitors for the treatment of HER2+ breast cancer patients and the combination of these inhibitors with HER2-targeted therapies [212]. However, early phase clinical trials of new immune agents for the treatment of patients with HER2+breast cancer have shown modest results.

Nanotechnology

One limitation that accounts for the compromised efficacy of ICB immunotherapy is off target binding of ICB antibodies to normal tissues upon systemic administration. Ideally, ICBs should be delivered selectively to the tumor site to avoid systemic non-specific activation of the Immune System resulting in irAEs (Immune-Related Adverse Effects). Immune checkpoint blockades have been reported to induce Inflammatory Side Effects (IRAEs), which resemble autoimmune disease [204]. Recent years have witnessed novel developments in pharmaceutical nanotechnology, or nanomedicine, as a possible strategy to ameliorate immunotherapy technical weaknesses. To improve the long-lasting response rate of checkpoint blockade therapy, nanotechnology has been employed at first for the delivery of single checkpoint inhibitors; while therapy via single immune checkpoint blockade results in resistance and a short period of response, strong interest has been raised to efficiently deliver immunomodulators targeting different inhibitory pathways or both inhibitory and costimulatory pathways. Promising results of immune checkpoint blockade therapy in combination with nanotechnology delivery systems have been reported [214]. nanotechnology, an interdisciplinary science, represents an effective tool to design highly effective combinational therapies boosting the effectiveness of immunotherapy and overpowering its limits (useful in overcoming critical points of Immune Checkpoint Inhibitors (ICIs) therapy such as the localized/targeted and controlled ICI release (availability) and ICI stability after infusion; further, they may allow for a reduction of ICI dose and control over Adverse Immune-Related Events (AIEs).

Reports have been given that although anti-CTLA4 treatment allowed for the overall survival of melanoma to be reached, with even cure of metastatic disease, for about 20% of

the patients; nevertheless, clinical application is limited because of its AIEs [215,216]. It has been suggested that CTLA-4 blockade can promote recruitment of peripheral T cells, thus enhancing the probability of autoimmune reaction [217]. Thus, the need for nanotechnological delivery of ICIs has presented itself which will enhance antibody bioavailability inside the tumor in order to improve the efficacy of the treatment, and at the same time limit the toxic effects due to the systemic exposure. Immunotherapy of HER2-positive breast cancer can benefit from the nanotechnology approach as it progresses to the clinic. NP-mediated ICI delivery seems an efficient and feasible approach to overcome the unsuccessful systemic administration of mAb against ICB in brain glioma treatment, due to drug failure in crossing the BBB. For this reason, combined nanotechnology and immunotherapy [218]. These authors covalently attached ICI mAb, such as anti-CTLA-4 (and/or anti-PD-1) to a biological polymeric scaffold made of Poly (-L-Malic Acid) (PMLA). These nanoscale immunoconjugates allowed the ICI mAb to go across the BBB and get to the cancer environment, where they modulated the immune response Mice bearing GBM showed an increase in survival when treated with nanocarriers loaded with anti-CTLA-4 (or anti-PD-1) with respect to the free mAb, and mice survival became even longer when treated with nanocarriers loaded with both ICI mAbs [218]. Moreover, mice bearing GBM showed a significantly longer survival when treated with double checkpoint inhibitor-bearing nanocarriers compared to those treated with single checkpoint inhibitors.

Nanotechnology is also useful in reducing drug dose as proposed by Schmid and colleagues [219]. They used Poly (Lactide-O-Glycolic) Acid (PLGA) and PEG NPs conjugated with anti-PD-1 mAb (PD-1 targeting NPs) and loaded with the Transforming Growth Factor- Receptor 1 (TGF- R1) inhibitor, SD-208, to target PD-1 cells and to block the immunosupressive activity of TGF-β in an MC38 colon cancer model. They showed that PD-1 targeting NPs-SD-208 acted on CD8+T cells, reduced tumor growth and ameliorated animal survival. The therapeutic effect was obtained at low doses of ICI (20 μg of anti-PD-1 and 40 μg of SD-208); whereas anti-PD-1 mAb and SD-208 in free administration did not have any effects. In a 4T1 tumor-bearing mice model used Iron-Oxide Nanoparticles (IONPs), which, after systemic administration, were strongly stable, and the small size favored their accumulation into the tumor site where they mediated hyperthermia after NIR irradiation. These authors suggested that local IONP-mediated photothermal therapy combined with ICI, such as anti-CTLA-4, can allow immunosuppression mediated by Treg to be overcome, thus boosting cancer immunotherapy [220].

Combining immunotherapy and multifunctional nanoprobes can achieve early cancer diagnosis and treatment [201]. Studies have shown that nanoparticle-assisted radiotherapy, chemotherapy, Photothermal Therapy (PTT) and Photodynamic Therapy (PDT) can stimulate the immune system by Inducing Immunogenic Cell Death (ICD) [221,222]. When combined with Immune Checkpoint Blockade (ICB), the localized therapies can activate tumor-specific immune response to target metastatic cancer cells, and induce immune memory to inhibit tumor recurrence [223,224]. Therefore, various therapeutic modalities can significantly augment the effects of immunotherapy against residual tumor cells. High-resolution imaging by multifunctional nanoparticles can also elucidate the mechanisms of immunotherapy [225,226] *via* real-time monitoring of immune cells in the Tumor Micro

Environment (TME) and the biodistribution of immunomodulatory drugs at the target site [227–230].

CONCLUSION

Nanotechnology platforms for the delivery of small molecule TKIs relied on EPR mechanism for delivery to tumors. It is our strong believe that small molecule TKIs can benefit from strategies that incorporate active targeting of tumor cells using large molecule TKIs (monoclonal antibody) as targeting moiety and which can exert therapeutic effects. Hence there is ample opportunity for various research groups to exploit the numerous targeted delivery techniques discussed in this review for enhancing the efficacy of TKIs in combination with other antineoplastic agents. Further, nanotechnology platforms can augment the delivery and enhance the efficacy of the new generation of treatment modality (immunotherapy) that stimulates the host immune system to selectively kill cancer cells with relatively fewer side effects on healthy tissues. The clinical progress made with immunotherapy has been spectacular, achieving complete cures and inducing long-term survival in advanced-stage patients. Unfortunately, however, immunotherapy only works well in relatively small subsets of patients. Increasing amounts of preclinical and clinical data demonstrate that combining nanomedicine with immunotherapy can boost therapeutic outcomes, by turning “cold” nonimmunoresponsive tumors and metastases into “hot” immunoresponsive lesions. In fact, reports have shown that Nano-immunotherapy can be realized via three different approaches, in which nanomedicines are used:

- to target cancer cells,
- to target the tumor immune microenvironment, and
- to target the peripheral immune system

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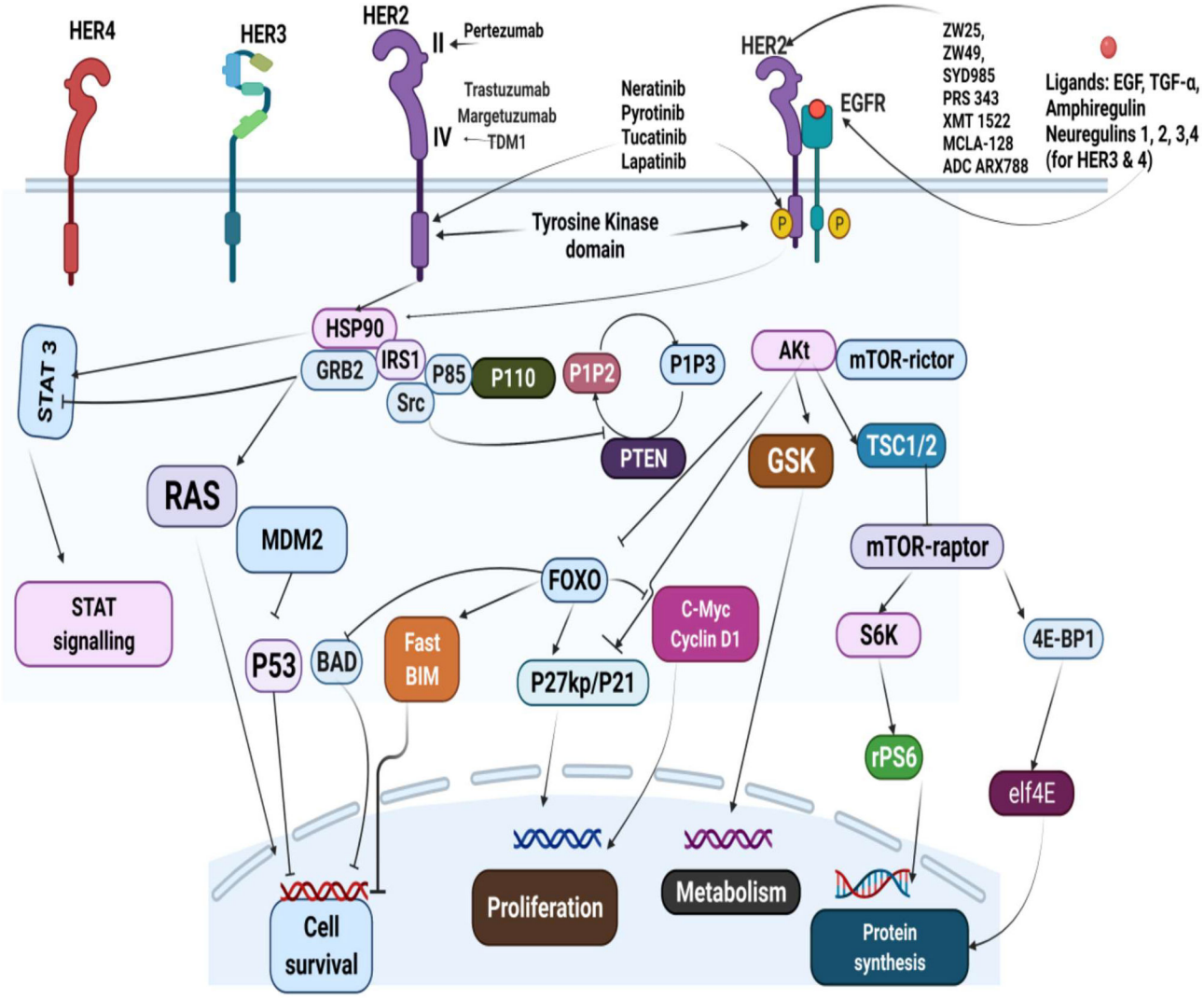

Figure 1). The ErbB family of receptors and site of action of anti-HER2 agents.

(EGFR Epidermal Growth Factor Receptor; P13K, phosphatidylinositol-3-kinase; PTEN Phosphatase and Tensin Homolog; GRB2 Growth Factor Receptor Bound. PIP3 Phosphatidylinositol-3,4,5- Diphosphate; MDM2, Murine Double Minute. BAD Bcl-2-Associated Death Promoter; FOXO, FORKHEAD homologin rhabdomyosarcoma; mTOR Mammalian Target of Rapamycin; STAT, Signal Transducer and Activator of Transcription; MAPK/ ERK Mitogen-Activated Protein Kinase/Extracellular Signal Regulated Kinase; HRG, Heregulin, Adapted from [44]).

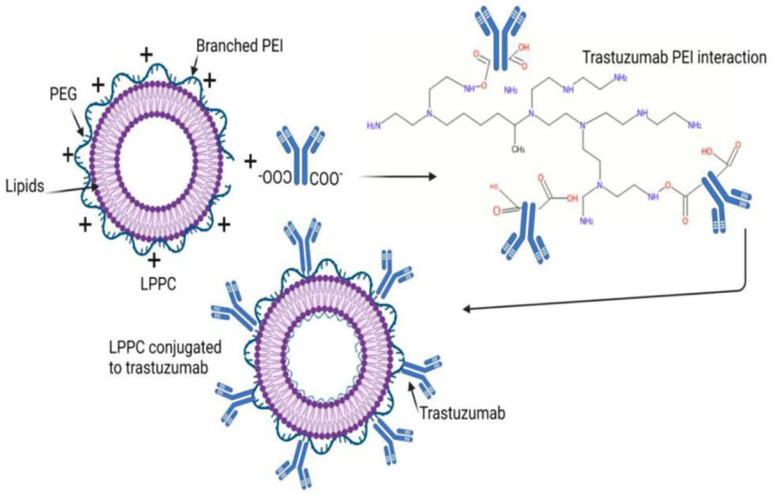

Figure 2). Synthesis of LPPC

Lipid-PEI-Cucurmin nanoparticle

PEG- Polyethylene glycol, PEI-Polyethylene imine. Adapted from [27]

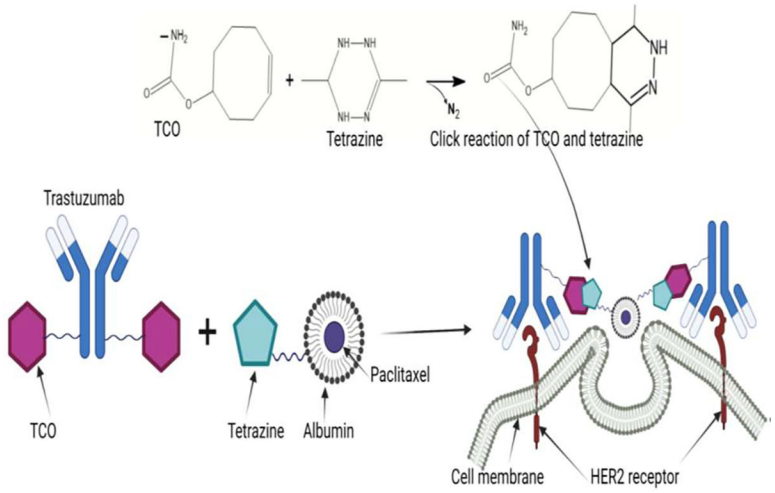

Figure 3). ***In vivo*** **click reaction between TCO and tetrazine at the HER2 receptor site** (TCO Trans-cyclooctene. Adapted from [5]).

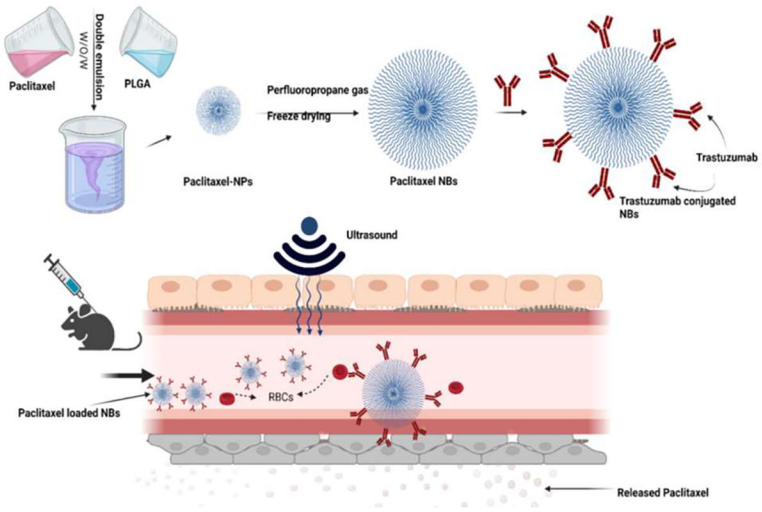

Figure 4). Synthesis and controlled release of paclitaxel nanobubbles via external ultrasound activation.

(NBs Nanobubbles; PLGA Polylactide-co-glycolide; NPs Nanoparticles; Adapted from [70]).

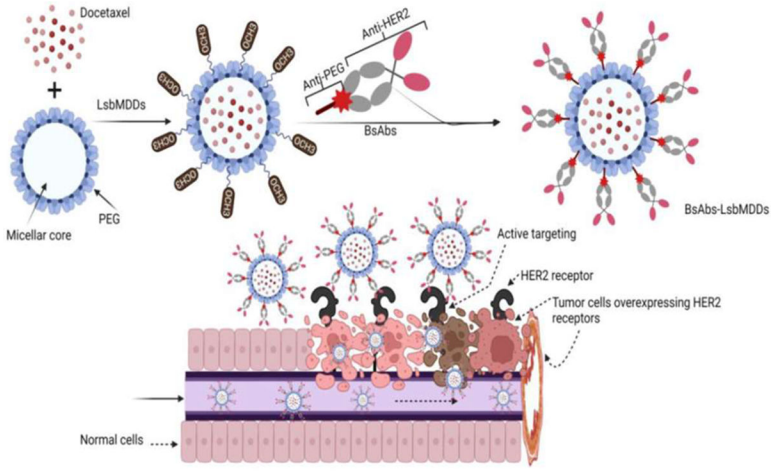

Figure 5). Schematics of the synthesis of BsAbs-LsbMDDs and targeting of HER2 receptors in tumour cells

BsAbs Bispecific antibody; LsbMDDs Lecithin stabilized micellar drug delivery system; PEG Polyethylene glycol; HER2 Human epithelial growth factor 2 receptor. Adapted from [134]

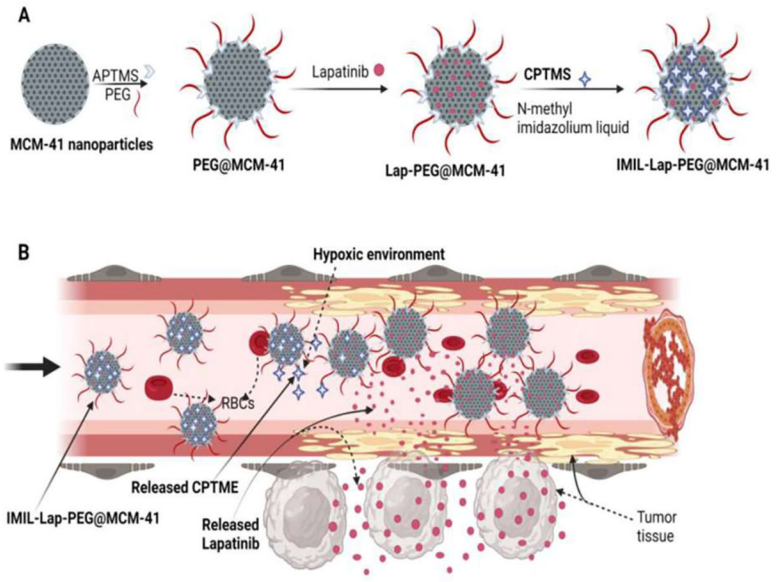

Figure 6).

Illustration of the **A)** Synthesis and drug loading of modified mesoporous nanoparticles **B)** Expected drug release model in vivo APTMS 3.(aminopropyl) trimethoxysilane CPTMS: 3-Chlororpropyltriethoxysilane; PEG: Polyethylene Glycol (PEG3000); MCM-41: Mesoposorous silicon nanoparticles. Adapted from [165]

TABLE 1

Novel ADCs and bispecific anti-HER2 mABs in preclinical and clinical trials

Name	Components	Target	Nano-Formulation	Mechanism	Trials	Outcomes	Reference
Novel ADCs							
SYD985	Trastuzumab duocarmazine	Domain IV of HER2	No	ADCC, alkylation	Phase I		[135,136]
					Phase III	ORR 33%, PFS 9.4 months in HER2 overexpressed tumors and 27%/40% in HR+ low	
						HER2 expression and HR− low	
						HER2 expression	
					Phase III TULIP NCT03262935	Expected	clinicaltrials.gov
MEDI4276	scFv trastuzumab/mAB 39S and AZ13599185 (tubulysin)	Domains IV and II of HER2	No	ADCC, inhibition of tubulin polymerization	Phase I	Substantial toxicity led to suspension for treatment of breast and lung cancers	[137]
ADCT-502	Trastuzumab, pyrolobenzodia zepine	HER2	No		Phase I	Trials terminated as efficacy fell below expectation (NCT03125200)	[42]
XMT 1522	Trastuzumab, auristatin F hydroxypropylamide		Yes	ADCC, alkylation		Trials halted due to perceived fatal outcome but lifted subsequently	[138
ADC ARX788	Dolostatin, HER2IgG	HER2	No	ADCC, alkylation	Phase I NCT03255070	Ongoing	133], clinicaltrials.gov
					NCT02512237		clinicaltrials.gov
					Phase II NCT04829604		clinicaltrials.gov
					NCT04983121,		clinicaltrials.gov
					NCT05018702		clinicaltrials.gov
5G9		trastuzumab optimalsynergistic epitope (TOSE)	No		Pre-clinical	Better uptake compared to pertuzumab. Superior synergistic outcome when combined with pertuzumab	[139]
Bispecific Antibodies							

Name	Components	Target	Nano-Formulation	Mechanism	Trials	Outcomes	Reference
MCLA-128	IgG1 BsAb	HER2, HER3	No	ADCC	Phase I/II NCT02912949 phase II NCT03321981	70% clinical benefit	[45,140]
					Phase II NCT03321981	Expected	clinicaltrials.gov
ZW25	Azymetric BsAb	Domains II/1V HER2	No	ADCC	Phase I	Promising outcome with no dose limiting toxicity	[45,141]
					NCT05035836	Ongoing	clinicaltrials.gov
					NCT04224272	-	clinicaltrials.gov
					NCT04276493	-	clinicaltrials.gov
ZW49	N-acyl sulfonamide auristatin, ZW25	HER2	No	ADCC, cytotoxic agent	-	-	[141]
GBR1302	2* HER2 +CD3 BsAb	HER2, CD3	No	ADCC	NCT03983395	Terminated due to business decision	[45]
PRS-343		HER2, CD137	No	ADCC	Phase 1 NCT03330561	Expected	[45,142]
					NCT03650348		clinicaltrials.gov
Tribody design	2*HER2+CD16 BsAb	HER2, CD16	No	ADCC via degranulation of immune cells	Preclinical	Superior effect in pancreatic ductal adenocarcinoma, breast cancer and autologous ovarian tumor cell lines	[143]