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Resistance mechanisms to anti-HER2 therapies in HER2-positive breast cancer: Current knowledge, new research directions and therapeutic perspectives

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ABSTRACT

HER2-positive breast cancer (HER2 + BC) represents 15–20% of all BCs. In the last two decades, the introduction of monoclonal antibodies (MoAbs), tyrosine kinase inhibitors (TKIs) and antibody-drug conjugates (ADCs) directed against HER2 impressively improved patient prognosis in all disease stages.

Yet, not all patients with limited-stage disease are cured, and HER2+ metastatic BC (mBC) remains an almost invariably deadly disease. Primary or acquired resistance to anti-HER2 therapies is responsible for most treatment failures. In recent years, several resistance mechanisms have been identified, such as impaired drug binding to HER2, constitutive activation of signaling pathways parallel or downstream of HER2, metabolic reprogramming or reduced immune system activation. However, only a few of them have been validated in clinical series; moreover, in the era of standard-of-care dual HER2 blockade, these mechanisms should be reassessed and, in case, confirmed with anti-HER2 combinations.

Defining the best strategies to delay or revert resistance to anti-HER2 treatments will be crucial to improve their clinical efficacy.

1. Introduction

Breast cancer (BC) harboring overexpression of the receptor tyrosine kinase (RTK) human epidermal growth factor receptor 2 (HER2) or amplification of the *HER2* gene, also referred to as HER2-positive (HER2 + ve) BC, accounts for about 15–20% of all BCs (Harbeck and Gnant, 2017). It is a highly aggressive neoplasm characterized by HER2-mediated activation of oncogenic pathways that drive cell cycle progression, angiogenesis, invasiveness and metabolic reprogramming, such as the Mitogen Activated Protein Kinase (MAPK) and the PI3K/AKT/mTOR cascades. Before the introduction of HER2-targeting therapies, the prognosis of patients with HER2 + ve metastatic BC (mBC) was especially poor as a result of fast tumor growth and lack of response to cytotoxic chemotherapy (ChT). In recent years, the availability of effective anti-HER2 agents has dramatically improved clinical outcomes in all disease stages.

The armamentarium of approved anti-HER2 compounds includes: trastuzumab (T), a humanized monoclonal antibody (MoAb) directed against HER2 ectodomain; pertuzumab (P), a MoAb that binds domain II of HER2, thus blocking its dimerization with other ErbB receptors, especially HER3; lapatinib (L), a selective, reversible, ATP-competitive tyrosine kinase inhibitor (TKI) of both HER2 and epidermal growth factor receptor (EGFR); and trastuzumab-DM1 (T-DM1), a conjugate of T and the anti-microtubule compound agent DM1 (derivate of maytansine).

Between 2000–2011, the combination of T or L with ChT provided first evidence of the effectiveness of HER2 inhibition (Slamon et al., 2001; Geyer et al., 2006; Andersson et al., 2011). More recently, taxane-based ChT plus dual HER2 blockade with T-P demonstrated unprecedented efficacy as a first-line treatment of HER2 + ve mBC (Baselga et al., 2012), while T-DM1 was more effective than L plus capecitabine after progression to T-based therapy (Verma et al., 2012).

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Overall, these therapeutic progresses have translated into higher cure rates of early-stage disease, as well as into impressive prolongation of patient progression free survival (PFS) and overall survival (OS) in the metastatic setting (Loibl and Gianni, 2017).

Despite these advancements, HER2 + ve mBC remains an almost invariably deadly disease, and the efficacy of individual anti-HER2 therapies is short-lived, especially for patients recurring after previous T-containing (neo)adjuvant treatment, with median PFS of about 1 year and less than 1 year in the first- and second-line settings, respectively (Ponde et al., 2018). While primary resistance to anti-HER2 agents is possible, most therapeutic failures derive from acquired resistance by sub-clones of cells that are progressively selected during the treatment. Different resistance mechanisms have been identified in preclinical studies, and some of them were preliminarily validated in clinical series. However, their reliability and clinical usefulness remain unclear.

Here we review the mechanisms implicated in primary or acquired resistance to single and dual HER2 blockade in HER2 + ve BC in both the preclinical and clinical setting, and we discuss possible strategies to translate recent discoveries into tangible clinical progresses.

2. Resistance to single anti-HER2 agents

2.1. Trastuzumab (T)

T has revolutionized HER2 + ve BC therapy, and actually represents the mainstay of treatment for HER2 + ve BC patients in all disease settings. It is a humanized murine MoAb that binds HER2 extracellular domain IV with high affinity and specificity (Carter et al., 1992). Mechanisms contributing to the antitumor activity of T can be divided in:

1) *Intracellular mechanisms*: by binding HER2 extracellular domain, T promotes its internalization and degradation (Cuello et al., 2001), prevents the formation of HER2-HER2 homodimers and HER2-HER3 heterodimers (Junttila et al., 2009), and inhibits HER2 ectodomain shedding that leads to the expression of the p95-HER2 isoform (Molina et al., 2001); these biological activities finally result in the inhibition of MAPK and PI3K/AKT/mTOR pathways downstream of HER2.

2) *Extracellular mechanisms*: T bound to HER2 on cancer cell membranes is recognized by Fcγ receptors expressed by cells of the innate immune system, including natural killer (NK) cells, antigen-presenting cells (APCs) and effector immune cells; this leads to clearance of T-bound cancer cells through two mechanisms that are known as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Clynes et al., 2000; Arnould et al., 2006).

Although T dramatically improved the prognosis of HER2 + ve mBC patients, approximately 75% of them progress within 12 months from T initiation (Slamon et al., 2001; Vogel et al., 2002). Different mechanisms responsible for *de novo* (primary) and acquired (secondary) resistance to T have been identified in preclinical studies, and some of them have been preliminarily validated in the clinical context (Tables 1–2).

2.1.1. Impaired binding of t to HER2

2.1.1.1. Low HER2 levels. The benchmark for considering BC as HER2 + ve is the overexpression of HER2 protein, as defined by an immunohistochemical (IHC) score of 3 + and/or the presence of *HER2* gene amplification by in situ hybridization (ISH) (Wolff et al., 2013). Several studies have clearly established a positive correlation between HER2 protein levels and tumor dependence on HER2 signaling, which predicts sensitivity to T: in particular, an IHC HER2 score of 3 + is associated with the highest sensitivity in both early-stage and advanced disease, followed by a score of 2 + and concomitant *HER2* amplification; conversely, most tumors with 1 + or 0 IHC scores are primarily resistant to T, and are excluded from anti-HER2 therapies (Vogel et al., 2002). However, IHC scores do not guarantee quantitative assessment of HER2 levels, which limits their ability to predict T sensitivity in individual patients. More reliable methods to quantify HER2 protein levels, such as mass spectrometry (MS) or methods based on fluorescent tags bound to anti-HER2 MoAbs, have been recently proposed to predict T efficacy (Nuciforo et al., 2016; Scaltriti et al., 2015). Intratumor heterogeneity of HER2 expression has also been associated with reduced activity of T-based treatments in the neoadjuvant setting, possibly reflecting the presence of cell clones that express low HER2 levels and are not HER2-dependent; these cells may progressively become dominant during exposure to T, which selectively targets HER2 + cells (Hou et al., 2017). Assessing HER2 heterogeneity may help in identifying tumors less likely to respond to T.

2.1.1.2. HER2 receptor variants or molecular masking. The expression of specific HER2 splicing variants may compromise the ability of T to bind HER2. The most studied variant is the p95HER2 isoform, an active, carboxy-terminal HER2 fragment deriving from HER2 ectodomain cleavage by the disintegrin/metalloproteinase ADAM10. The lack of the extracellular domain in p95HER2 makes it unable to bind T, and initial studies associated p95HER2 expression to resistance to T but not to L, which binds the intracellular portion of the receptor (Scaltriti et al., 2007, 2010; Sperinde et al., 2010). Disappointingly, one recent report correlated p95HER2 expression with higher benefit from T-containing neoadjuvant ChT (Loibl et al., 2011). Due to inter-study heterogeneity in methods used to detect p95HER2, and since the use of concomitant ChT can confound result interpretation, the predictive role of p95HER2 remains unclear.

Recent studies in BC cell lines identified a splicing variant of *HER2* lacking exon-16, namely HER2Δ16, which forms stable HER2 dimers in SRC-dependent manner (Castiglioni et al., 2006) and correlates with in vitro resistance to T (Mitra et al., 2009). However, experiments in transgenic mouse models revealed enhanced T sensitivity of HER2Δ16-expressing BCs when compared to wild-type *HER2*-expressing ones (Castagnoli et al., 2014; Palladini et al., 2017).

Due to these contradictory findings, the impact of HER2 splicing variants on T resistance remains uncertain. In particular, the prognostic/predictive role of specific variants could be context-specific – i.e. it may depend on disease stage, the type of treatment administered or biological tumor characteristics.

Another proposed mechanism of impaired binding of T to HER2 consists in the expression of the membrane-associated mucin 4 (MUC4) by tumor cells or cells in the microenvironment. Notably, MUC4 masks T binding site on HER2, thus making it unable to bind and inhibit HER2. However, the potential role of MUC4 in mediating T resistance needs to be better defined (Nagy et al., 2005).

2.1.2. Altered intracellular signaling

2.1.2.1. Constitutive activation of the PI3K/AKT/mTOR pathway. Since T-induced inhibition of the PI3K/AKT/mTOR signaling cascade is crucial for its anticancer activity, constitutive activation of the PI3K/AKT/mTOR axis can bypass its inhibition by T. In two preclinical studies, E545 K and H1047R activating mutations of PI3K catalytic subunit α (*PI3KCA*) were associated with T resistance in HER2 + ve BC models (Berns et al., 2007; Kataoka et al., 2010; Chandarlapaty et al., 2012). Similar results emerged in tumors expressing low levels of PTEN, a tumor suppressor phosphatase that reverts PI3K-induced phosphorylation of inositide lipids (Nagata et al., 2004; Dave et al., 2011). Consistent with these findings, pharmacological inhibitors of PIK3CA (copanlisib, alpelisib) or mTOR (everolimus) sensitize cancer cell lines to T (Elster et al., 2015; O'Brien et al., 2014; Lu et al., 2007). Notably, about 30% of human HER2 + ve BCs bear somatic alterations in PI3K/AKT/mTOR pathway genes, with *PIK3CA* mutations occurring in 20–23% of primary tumors (Loibl et al., 2016). Based on promising preclinical evidences, the phase III randomized BOLERO-1 and BOLERO-3 trials investigated the efficacy of adding everolimus to paclitaxel-T or vinorelbine-T in HER2 + vs mBC (Andre et al., 2014; Hurvitz et al., 2015a). Although everolimus failed to improve PFS in the whole patient population, the presence of alterations associated with

Table 1
Mechanisms of resistance to anti-HER2 agents.

Anti HER2 agent(s)	Mechanism of resistance	Factors involved
Trastuzumab (T)	Impaired HER2 binding	Low HER2 levels
	Parallel/downstream pathways	Splicing variants (p95HER2; Δ16 HER2)
	Enhanced lipid metabolism	PI3KCA mutations, PTEN loss
	ER signaling	FASN
	Cell cycle regulation	ER-PgR expression
	Escape from ADCC	Cyclin D1-CDK 4/6 expression
		Poor binding to CD16A
Lapatinib (L)	HER2 signaling	*HER2* mutations
	Cell cycle regulation	Cyclin D1-CDK 4/6 expression
	Parallel/Downstream pathways	PI3K/AKT/mTOR pathway alterations
	ER signaling	ER-PgR expression
T-DM1	Impaired HER2 binding	p95HER2; MUC4 expression
	Parallel/downstream signaling	NRG, HER2-HER3, *PIK3CA* mutations
	T-DM1 internalization/release	SLC46A3, MDR1
Trastuzumab plus Lapatinib (T + L)	Impaired HER2 binding	Low HER2 levels
	FGFR1 signaling	*HER2* mutations
	Downstream pathways	*FGFR1* amplification
	ER signaling	*PI3KCA* mutations,
	Cell cycle regulation	ER-PgR expression
		Cyclin D1-CDK 4/6 expression
Trastuzumab plus Pertuzumab (T + P)	Altered intracellular pathways	*PIK3CA* mutations
	HER2 signaling	*HER2* mutations

PI3K/AKT/mTOR pathway activation (mostly *PIK3CA* activating mutations) predicted benefit from everolimus (Andre et al., 2016).

Other intracellular mechanisms potentially involved in resistance to T consist in: a) aberrant activation of the tyrosine kinase SRC, which is placed downstream of several RTKs, including HER2; notably, SRC inhibition restores in vitro and in vivo sensitivity to T (Zhang et al., 2011); b) overexpression of Cyclin E, which binds cyclin-dependent kinase 2 (CDK2) and promotes the G1/S cell cycle transition. Interestingly, cyclin E is overexpressed in about 20% of HER2 + ve BC and is associated with worse patient prognosis. Based on these evidences, CDK2 inhibitors promise to improve the efficacy of anti-HER2 treatments (Scaltriti et al., 2011).

2.1.2.2. Cross-signaling to HER2

HER2 heterodimers. Therapeutic concentrations of T prevent HER2 homodimerization and inhibit HER2-driven signaling (Ghosh et al., 2011). However, different RTKs are able to heterodimerize with HER2, and to activate downstream signaling pathways similarly to HER2 homodimers. In particular, EGFR/HER1 and HER3 overexpression promotes the formation of HER2-EGFR and HER2-HER3 heterodimers, respectively; this allows tumor cells to use the few HER2 molecules that are not bound to T to reactivate HER2 signaling (Hellyer et al., 2001; Sergina et al., 2007; Ritter et al., 2007). Other HER2 dimerization partners include: insulin-like growth factor-1 receptors 1 and 2 (IGFR 1/2), the hepatocyte growth factor (HGF) receptor c-MET, EphA2 (Lu et al., 2001; Shattuck et al., 2008; Zhuang et al., 2010; Wang et al., 2011).

ER expression. About 50% of HER2 + ve BCs express ER, and are referred to as HER2 + ve ER-positive (ER + ve) BCs. They represent a distinct entity when compared to HER2 + ve ER-negative (ER-ve) tumors (Bianchini et al., 2011), and are characterized by lack of responsiveness to endocrine therapy (ET) (De Laurentiis et al., 2005) or T (Tortora, 2011) when used alone, as a result of bidirectional crosstalk between ER and HER2 pathways (Arpino et al., 2008). Conversely, combining ET with anti-HER2 therapies improved clinical outcomes in patients with HER2 + ve ER + ve BC, thus confirming the importance of inhibiting both signaling pathways (Kaufman et al., 2009; Johnston et al., 2009; Arpino et al., 2017; Johnston et al., 2018). Despite these evidences, taxane-based chemotherapy plus anti-HER2 agents still remains the mainstay of treatment also for patients with HER2 + ve ER + ve mBC.

2.1.2.3. Activation of cyclinD1-cyclin-dependent kinase 4/6 (CDK 4/6) axis. The cyclin D1-CDK4/6 axis is crucially implicated in the G1-S cell cycle transition, and enhanced activation of this axis, as mediated by cyclin D1/CDK4 overexpression or *CDK4* mutations, causes resistance to ET in ER + ve BC (Sledge et al., 2017; Cristofanilli et al., 2016). The following evidences also point to the role of Cyclin D1-CDK 4/6 axis in T resistance in HER2 + ve BC: a) the inability to express cyclin D1 in mammary glands protects mice from developing HER2-driven tumors (Landis et al., 2006); b) the CDK 4/6 inhibitor palbociclib synergizes with T against different HER2 + ve BC lines (Finn et al., 2009); c) transgenic mouse models of HER2 + ve BC select cyclin D1 and CDK4 overexpression upon HER2 downregulation, while combining T with the CDK 4/6 inhibitor abemaciclib produces synergistic anticancer activity against in vitro and in vivo HER2 + ve BC models (Goel et al., 2016). *Cyclin D1* gene overexpression has also been associated with lower pathological complete response (pCR) rates in patients receiving T-containing neoadjuvant ChT (Goel et al., 2016). Finally, neoadjuvant, chemotherapy-free T-P-palbociclib-fulvestrant quadruple combination has recently demonstrated promising activity, in terms of precocious reduction of ki-67, as well as of clinical and pathologic complete responses, against HER2 + ve ER + ve BC (Gianni et al., 2018); in particular, results of this study provide clinical proof of concept that activation of the cyclin D1-CDK 4/6 axis may limit the in vivo antitumor activity of ER-HER2 inhibition, while targeting CDK 4/6 in combination with HER2 inhibitors and endocrine treatments may offer a valid alternative to ChT-containing regimens against triple-positive BC, at least in some patients. Based on these promising results, T-palbociclib ± letrozole is now being explored in patients with HER2 + ve ER + ve/ER-ve mBC (NCT02448420 trial, Table 3).

Fatty acid synthase (FASN) overexpression. Neoplastic cells *de novo* synthesize their lipids from glucose-derived acetyl-CoA units, and the fatty acid synthase (FASN) enzyme, which elongates nascent fatty acid chains, plays a crucial role in this process (Vernieri et al., 2016). Of note, FASN is frequently overexpressed in HER2 + ve BC, and is associated with worse clinical outcomes (Vernieri et al., 2016). Compelling preclinical evidences indicate a cross-regulation between HER2 and FASN; indeed, HER2 stimulates FASN expression and post-translational activation (through phosphorylation), and FASN promotes HER2 localization in lipid rafts on the plasma membrane and HER2 signaling (Menendez and Lupu, 2007). Moreover, FASN overexpression causes resistance to T in HER2 + ve BC preclinical models, while pharmacological blockade of FASN inhibits HER2 synergistically with

Table 2
New drugs to overcome resistance to anti-HER2 agents – preclinical and clinical evidences.

MECHANISM OF RESISTANCE	ALTERATED PATHWAY	TARGET THERAPY	RESISTANCE TO	PRECLINICALEVIDENCES	CLINICAL RESULTS
Independent activation of intracellular signaling	PI3K/AKT/ mTOR	**Dactolisib (D)**	T/L	**D ± T or L** *Eichhorn PJA, 2008* (Eichhorn et al. (2008) **D ± T or L (**Serra V, 2008) (Serra et al. (2008))	**NA**
		Copanlisib (C)	T/L	**C ± T or L** Elster, (2015) (Elster et al., 2015)	**NA**
		Everolimus (E)	T	**E + T** Hurvitz, (2015) (Hurvitz et al. (2015b))	**Phase 1: E + L in advanced solid tumors** *Gadgeel SM, 2013* (Gadgeel et al. (2013)) MTD L 1250 + 5 mg E daily A patient with breast cancer achieved an unconfirmed PR (ER + ve, PR − ve, Her2 unknown) **Phase 1/2: E + T in pre-treated mBC** *Morrow PK, 2011* (Morrow et al. (2011)) ORR 15% CBR 34% PFS 4.1 mos **Phase 2: Sirolimus + T in pre-treated mBC** *Acevedo-Gadea C, 2015* ORR 11% CBR 44 % **Phase 2b: Ridafolimus + T in pre-treated mBC** *Seiler M, 2015* (Acevedo-Gadea et al. (2015)) ORR 15% CBR 34.3% PFS 5.4 mos (range 0-20.3 mos; 95% CI, 2.0-7.4 mos) OS 17.7 mos (range, 0-25.9 mos; 95% CI, 8.8-20.8 mos) PFS (6 mos) 37% **Phase 3: E + T + Vinorelbine (V) vs T + V in pre-treated pts with Paclitaxel (Px)** *Andrè F, 2014 (Andre et al., 2014)* PFS 7 vs 5.78 mos HR 0.78 (95% CI 0.65-0.95; p = 0.0067) **Phase 3: I line E + T + Px vs T + Px** *Hurvitz SA, 2015* (Hurvitz et al., 2015a) Overall population (n = 480) PFS 14.95 vs 14.49 mos HR 0.89 (95% CI 0.73-1.08; NS). HHRR-negative subpopulation (n = 311) PFS 2.27 vs 13.08 mos HR 0.66 (95% CI 0.48-0.91; NS)
		Pictilisib (Pi)	T-DM1	**Pi + T-DM1** *Li G, 2018 (Li et al., 2018)*	**NA**
		Alpelisib (BYL-719)	T/T-DM1	**BYL-719 + T** *O'Brien, 2014* (O'Brien et al., 2014)	**Phase 1: BYL-719 + T-DM1** *Jain S, 2018* (Jain et al. (2018) ORR 43% in overall population (n = 17) ORR 30% in T-DM1-resistant pts (n = 10)
	ErbB2 mutations	**Tesevatinib (Te)**	L	**Te** *Trowe T, 2008 (Trowe et al., 2008)*	**NA**
	p70S6K1 activation	**Rapamycin (R)**	L	**R + L** *Vasquez-Martin A, 2008* (Vazquez-Martin et al. (2008)) **R ± L** *Gayle SS, 2012* (Gayle et al. (2012))	**NA**
	AXL activation	**Foretinib (F) or anti-estrogenic (AE) therapies**	L	**F or AE ± L** *Liu L, 2009* (Liu et al. (2009))	**NA**
	SRC activation	**Saracatinib (S)**	T	**S + T** *Zhang S, 2011* (Zhang et al. (2011)	**NA**
		Saracatinib/ Dasatinib (D)	L	**D or S ± L** *Rexer BN, 2011* (Rexer et al. (2011))	
		Saracatinib/ Cetuximab (Cx)	L	**S or Cx ± L** *Formisano L, 2014* (Formisano et al. (2014))	
	mTOR- dependent upregulation of IAPs	**AZD8055 (A) or Tanespimicin (Tan)**	L	**A or Tan ± L** *Brady SW, 2015* (Brady et al. (2015))	**NA**
	Amplification of FGFR signaling	**Lucitanib (Lu)**	T + L	**L ± (T + L)** *Hanker AB, 2017* (Hanker et al. (2017))	**NA**

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Table 2 (*continued*)

MECHANISM OF RESISTANCE	ALTERATED PATHWAY	TARGET THERAPY	RESISTANCE TO	PRECLINICALEVIDENCES	CLINICAL RESULTS
	ER pathway	**Anti-estrogen (AE)**	T + L	**AE ± (T + L)** *Wang Y-C, 2011* (Wang et al. (2011))	**Phase 2: Neoadjuvant T** + L ± **Letrozole (Letro) in stage II/III BC HHRR** +/- *Rimawi MF, 2013* (Rimawi et al. (2013) pCR 27% in overall population pCR 21% in ER + ve treated with T + L + Letro pCR 36% in ER -ve treated with T + L **Phase 3: I/II line Aromatase Inhibitors (AI) + (T + L) vs AI + T vs AI** + L **in T and AI-pretreated mBC** *Johnston SRD, 2018 (Johnston et al., 2018)* PFS 11 vs 5.7vs 8.3 mos HR AI + (T + L) vs AI + T 0.62 (95% CI, 0.45-0.88, P = .0064) HR AI + L vs AI + T 0.71(95% CI, 0.51-0.98, P = .0361) ORR 31.7 vs 13.7 vs 18.6% OS 46.0 vs 40.0 vs 45.1 mos HR AI + (T + L) vs AI + T 0.60 (95%CI, 0.35-1.04) HR AI + L vs AI + T 0.82
Impaired ADCC	NF-κB signalling	**IKKβ inhibitor (IMD-0354)**	L	**IMD-0354 ± L** *Wetterskog D, 2014* (Wetterskog et al. (2014)	**NA**
	Lacking of CD16A and/or overexpression of CD32B	**Margetuximab (M)**	T	**M** *Nordstrom JL, 2011* (Nordstrom et al. (2011)	**Phase 1: pretreated HER2 amplified gastric or breast M 0.1–6.0 mg/kg for 3 of every 4 weeks (Regimen A) or once 10–18 mg/kg every 3 weeks (Regimen B)** *Bang YJ, 2017* (Bang et al. (2017) ORR 12% regimen A, 50% regimen B
	TNFα-induced MUC4 upregulation	**Etanercept (Et)**	T/T-DM1	**Et ± T** *Mercogliano MF, 2017* (Mercogliano et al. (2017)	**NA**
	Impaired lymphocyte-mediated cellular cytotoxicity	**Anti-CD137 and anti–PD-1**	T	**Anti-CD137 and anti–PD-1 + T** *Stagg G, 2011* (Stagg et al. (2011))	**Phase 1b/2: Pembrolizumab + T in T-resistant HER2-positive mBC** *Loi S, SABCS 2017 (Loi et al., 2017)* ORR 15.2% DCR 24% mDOR 11.1 months in PD-L1 +ve ORR 39% and DCR 47% in PD-L1 + ve ≥ 5% TILs. No response in PD-L1-ve.
Impaired cell cycle regulation	CCNE1 overexpression	**CYC065 (Cy)**	T	**Cy ± T** *Scaltriti M, 2011* (Scaltriti et al. (2011)	**NA**
Fatty acid biosynthesis	FASN overexpression	**C75**	T	**C75 ± T** *Vazquez-Martin A, 2007* (Vazquez-Martin et al. (2007))	**TVB-2640 + paclitaxel** Brenner A. 2017 (Brenner et al., 2017)

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Table 2 (*continued*)

MECHANISM OF RESISTANCE	ALTERATED PATHWAY	TARGET THERAPY	RESISTANCE TO	PRECLINICALEVIDENCES	CLINICAL RESULTS
Cross-Signaling to HER2	Heregulin-EGFR-HER3 autocrine signaling axis	**Neratinib (N)**	L	**N** *Xia W, 2013* (Xia et al. (2013))	**Phase 1: N + T +Px in mBC** *Jankowitz RC, 2013* (Jankowitz et al. (2013)) ORR 38 % CBR ≥ 24 weeks 52 % PFS 3.7 mos **Phase 1: N + Temsirolimus (Tem) in advanced solid tumors** *Gandhi L, 2014* (Gandhi et al. (2014)) MTD N/Tem 200/25 mg or 160/50 mg ORR 16% in overall population 22% (2/9) in HER2-amplified BC CBR 71% in overall population **Phase 2: N + V in pre-treated mBC** *Awada A, 2013* (Awada et al. (2013)) ORR 41% in overall population 8% in pretreated with L **Phase 2: N + Px in pre-treated mBC** *Chow LW, 2013* (Chow et al. (2013)) ORR 73% in overall population (95% CI, 62.9-81.2%) 71% in pretreated with 0/1 line 77 in pretreated with 2/3 lines (L admitted) PFS 57.0 weeks (95% CI, 47.7-81.6 weeks) **Phase 2: N + Capecitabine (Cape) in T-pre-treated mBC** *Saura C, 2014* (Saura et al. (2014)) ORR 64% (95% CI, 51% to 76%) in L-naive 57% (95% CI, 18% to 90%) previous L PFS 40.3 ws (95% CI, 30.3-66.0 ws) in L-naive 35.9 ws (95% CI, 18.9-60.1 ws) previous L **Phase 2 non-inferiority: N vs L + Cape in pre-treated mBC** *Martin M, 2013* (Martin et al. (2013)) PFS 4.5 vs 6.8 mos HR 1.19 (95% CI, 0.89–1.60; non-inferiority margin 1.15) OS 19.7 vs 23.6 mos ORR 29 vs, 41% (NS) CBR 44% vs 64% (P = 0.003) **Phase 3: I line N + Px vs T + Px** *Awada A, 2016* (Awada et al. (2016)) PFS 12.9 (95%CI, 11.1-14.9) vs 12.9 (95%CI, 11.1-14.8) months HR 1.02 (95% CI, 0.81-1.27, NS) OS (cut-off date) 32.2 vs 30.4% HR, 1.05 (95% CI, 0.76-1.45, NS ORR 74.8 vs 77.6% (NS) CBR 88.4 vs 85.2% (NS)
		Pertuzumab (P)	T-DM1	**P ± T-DM1** *Phillips GD, 2014 (Phillips et al., (2014)*	**Phase 3: I line T + taxane vs T-DM1 vs T-DM1 + P** *Perez EA 2017* (Perez et al. (2017) PFS 13.7 vs 14.1 vs 15.2 months HR for: - T-DM1 vs T + taxane: 0.91 (97.5% CI, 0.73-1.13, NS) - T-DM1 + P vs T + taxane: 0.87 (97.5% CI, 0.69-1.08, NS 97.5% CI non-inferiority margin 1.18) - T-DM1 + P vs T-DM1: 0.91 (97.5% CI, 0.73-1.13, NS) ORR 67.9% (195/287; 95% CI, 62.3-73.3%) vs 59.7% (181/303; 95% CI, 54.1-65.3%) vs 64.2% (192/299; 95% CI, 58.6-69.7%)
Upregulation of ABC	Increased MDR1	**XR9051 (X)**	T-DM1	**X + T-DM1** *Li G, 2018 (Li et al., 2018)*	**NA**

T, and halts cancer cell proliferation/survival (Vazquez-Martin et al., 2007). Based on results of two phase I trials showing good tolerability and promising anticancer activity of the selective FASN inhibitor TVB 2640 in patients with solid neoplasms (Brenner et al., 2017; Patel et al., 2015), one phase II study is currently investigating the ability of TVB 2640 to restore T sensitivity in patients with T-pre-treated HER2+ve mBCs (NCT03179904 trial; Table 3).

2.1.3. Escape from ADCC

The Fc portion of T stimulates ADCC by binding the activating

Table 3
Ongoing trials with new anti-HER2 agents and combinations in HER2+ mBC.

Agent	Mechanism of action	Ongoing trials
New anti-HER2 antibodies		
Margetuximab (MGAH22)	Chimeric IgG mAb with high affinity for CD16A polymorphisms and low affinity for FcγRIIB (CD16B) of HER2	Phase 3 SOPHIA (NCT02492711): margetuximab + ChT vs trastuzumab + ChT
MCLA 128	IgG1 biospecific antibody targeting HER2 and HER3	Phase 1/2 (NCT02912949) Phase 2 (NCT03321981): MCLA-128 + trastuzumab with or without ChT (vinorelbine)
ZW 25	Humanized bi-specific antibody directed against two distinct epitopes of HER2	Phase 1 (NCT02892123): ZW25 with or without ChT (paclitaxel, capecitabine or vinorelbine)
Antibody-Drug Conjugate (ADC)		
DS 8201	Humanized anti-HER2 Ab enzymatically linked to deruxtecan (novel topoisomerase I inhibitor)	Phase 1 (NCT02564900): DS8201 in TDM-1 pretreated pts Phase 1 (NCT03368196): dose finding in Chinese pts Phase 1 (NCT03366428): Assessment of effect of DS8201 on the QTc Phase 2 (NCT03248492): DS8201 in T-DM1 resistant or refractory pts; exploratory for TDM-1 intolerant pts
SYD 985 ([vic-]trastuzumab duocarmazine)	ADC based on trastuzumab and a cleavable linker-duocarmycin (alkylant product, vc-seco-DUBA) payload	Phase 3 TULIP (NCT03262935): SYD985 vs TPC in TDM-1 resistant pts Phase 1 (NCT02277717): dose finding
Tyrosine kinase inhibitors (TKIs)		
Neratinib	Potent, irreversible, TKI of HER1, HER2 and HER4	Phase 3 NALA (NCT01808573): neratinib + capecitabine vs. lapatinib + capecitabine Phase 2 (NCT01670877): neratinib + fulvestrant in HER2 non-amplified but HER2 mutant mBC Phase 1/2 (NCT02236000): neratinib + T-DM1 in T-DM1 naïve pts Phase 1b (NCT03101748): neratinib + pertuzumab, + trastuzumab + paclitaxel in treatment naïve mBC Phase 1/2 (NCT03377387): capecitabine 7/7 Schedule + neratinib in heavly pretreated mBC Phase 2 (NCT03289039): neratinib + fulvestrant in ER+/HER2+, trastuzumab-resistant mBC
Tucatinib	Potent, ATP competitive TKI that is selective for HER2 without inhibition of EGFR	Phase 1/2 (NCT03054363): tucatinib + palbociclib + letrozole in ER+HER2+ mBC Phase 2 (NCT02614794): tucatinib/placebo + trastuzumab + capecitabine in trastuzumab, pertuzumab, and T-DM1 pretreated pts Phase 2 (NCT03501979): tucatinib + trastuzumab + capecitabine in pts with leptomeninngeal metastases
Poziotinib	Quinazoline-based, small-molecular and irreversible pan-HER TKI	Phase 2 (NCT02544997): poziotinib in pretreated pts with HER2 or EGFR mutation Phase 1 (NCT03429101): poziotinib + T-DM1 Phase 2 (NCT02659514): poziotinib in trastuzumab and TDM-1 pretreated pts
Pyrotinib	Irreversible dual EGFR/HER2 TKI	Phase 1 (NCT02500199): dose finding Phase 3 (NCT03080805): pyrotinib + capecitabine vs. lapatinib + capecitabine Phase 2 (NCT03412383): pyrotinib in HER2 Non-amplified but HER2 Mutant pts
Immune checkpoint inhibitors		
Atezolizumab	Fully humanized IgG1 mAb against PD-L1	Phase 2 (NCT03417544): atezolizumab + Trastuzumab + Pertuzumab in CNS mets pts Phase 2 (NCT03125928): atezolizumab + Trastuzumab + Pertuzumab + paclitaxel in first line HER2+ mBC Phase 2 KATE2 (NCT02924883): atezolizumab + T-DM1 vs. atezolizumab + placebo
Pembrolizumab	Humanized IgG4 mAb against PD-1	Phase 3 (NCT03199885): pembrolizumab + trastuzumab + pertuzumab + paclitaxel in first line HER2+ mBC Phase 1 (NCT03032107): pembrolizumab + T-DM1
Durvalumab	Fully humanized IgG1 mAb against PD-L1	Phase 1b (NCT02649686): durvalumab + trastuzumab in trastuzumab and pertuzumab-pretreated pts (preferably alsoT-DM1 pretreated)
CDK 4/6 inhibitors		
Palbociclib	CDK 4/6 inhibitor	Phase 1/2 (NCT03304080): anastrozole + palbociclib + trastuzumab + pertuzumab in first line ER+HER2+ mBC Phase 2 PATRICIA (NCT02448420): palbociclib + trastuzumab ± letrozole in trastuzumab resistant pts Phase 1/2 (NCT03054363): tucatinib + palbociclib + letrozole in ER+HER2+ mBC Phase 3 PATINA (NCT02947685): palbociclib + anti-HER2 Therapy + ET vs. anti-HER2 Therapy + ET after induction treatment (trastuzumab + ChT) Phase 2 (NCT02774681): palbociclib + trastuzumab in CNS mets pts
Ribociclib	CDK 4/6 inhibitor	Phase 1/2 (NCT02657343): ribociclib + trastuzumab or T-DM1 in trastuzumab, pertuzumab and T-DM1 resistant pts
Abemaciclib	CDK 4/6 inhibitor	Phase 2 monarcHER (NCT02675231): abemaciclib + trastuzumab ± fulvestrant vs. TPC (trastuzumab + ChT) in trastuzumab and T-DM1 resistant pts
PI3K/mTOR inhibitors		
Alpelisib (BYL 719)	α-specific Pi3K inhibitor	Phase 1 (NCT02167854): LJM716, alpelisib and trastuzumab in heavily pretreated pts
Copanlisib (BAY 80-6946)	Pan-class I PI3K inhibitor with predominant activity against PI3K -α and –δ isoforms	Phase 1 panHER (NCT02705859): copanlisib + trastuzumab in heavily pretreated pts
Taselisib	β-specific PI3K inhibitor	
Pictilisib (GDC 0941)	Pan-class I PI3K inhibitor with predominant activity against PI3K -α and –δ isoforms	Phase 1 (NCT00928330) pictilisib + trastuzumab or T-DM1
Everolimus		
FASN inhibitors		
TVB 2640	Small-molecule reversible inhibitor of FASN (fatty acid synthase).	Phase 2 (NCT03179904): TVB-2640 + paclitaxel + trastuzumab in pts resistant to trastuzumab-Taxane-Based Therapy

antibody receptor CD16A: FcγRIII on myeloid cells, while T binding to the inhibitory receptor CD32B: FcγRIIB on the same cells prevents ADCC. Notably, mice lacking CD16A have impaired ADCC-mediated lysis of cancer cells, and HER2 + ve tumors growing in these animals are resistant to T (Clynes et al., 2000). The recently synthesized MoAb margetuximab (MGAH22), which contains an Fc domain that binds CD16A more efficiently and CD32B more weakly than T, induced ADCC against T-resistant HER2 + ve BC cells (Nordstrom et al., 2011). In a phase I, first-in-human clinical trial, margentuximab demonstrated anticancer activity against 100% (n = 24) of HER2 + ve mBC with acquired resistance to T, and induced ex-vivo ADCC more efficiently than T (Bang et al., 2017). Based on these results, margentuximab is being investigated in the context of phase III trials (Table 3)

Overexpression of Neuromedin U (NmU), which stimulates TGFβ1 and PD-L1 secretion in tumor microenvironment, has also been associated with impaired ADCC and less effective antitumor immune response (Martinez et al., 2017). In NmU-overexpressing tumors, the use of immune checkpoint inhibitors could prevent or revert T resistance. In this respect, the phase Ib/II PANACEA trial recently showed a 15.2% ORR in unselected patients with T-resistant disease treated with the anti PD-1 MoAb Pembrolizumab plus T (Loi et al., 2017).

2.2. Lapatinib (L)

L is a synthetic, oral TKI that reversibly binds to the cytoplasmic ATP-binding site of both HER2 and EGFR, thus preventing HER2 phosphorylation and activation (Rusnak et al., 2001). In combination with capecitabine, L improved the survival of HER2 + ve mBC patients who had received prior T-containing therapy (Medina and Goodin, 2008), and is a standard third-line treatment option. However, some tumors are primarily resistant to L, and the remaining ones acquire resistance after a median time of 6 months (Verma et al., 2012). The most studied mechanisms of resistance consist in: *HER2* mutations; activation of signaling cascades parallel to HER2; activation of pathways downstream of HER2 (Tables 1–2).

2.2.1. HER2 gene mutations

Single amino acid substitutions in HER2 regions responsible for kinase activation can impair the ability of L to bind HER2. One study identified 17 different HER2 amino acid substitutions associated with L resistance, with the *HER2* L755S and T798I mutations causing the highest levels of resistance. In particular, the *HER2* T798I mutation has similar clinical relevance to the *EGFR* T790 M mutation in non-small cell lung cancer that progresses during therapy with the first-generation EGFR inhibitors gefitinib and erlotinib (Trowe et al., 2008). More recently, the *HER2* mutation T798 M was associated with resistance to L but not to the irreversible HER2/EGFR TKI afatinib (Rexer et al., 2013).

Of note, the EGFR/HER2/vascular endothelial growth factor receptor (VEGFR) inhibitor EXEL-7647, which binds HER2 in both its active and inactive conformations, is able to target most known *HER2* mutations, including HER2 T798I (Trowe et al., 2008; Gendreau et al., 2007). In addition, the irreversible pan-HER TKI neratinib recently demonstrated anticancer activity in patients with mBCs harboring mutations in the HER2 TK domain (including HER2 L755), independently from HER2 expression levels (Hyman et al., 2018). Therefore, neratinib could be active against some L-resistant HER2 + ve mBC. Ongoing randomized trials are comparing L-capecitabine with neratinib-capecitabine in patients progressing after T- and T-DM1-based treatments (Table 3).

2.2.2. Activation of pathways parallel to HER2

2.2.2.1. Increased expression of RTK ligands. Increased extracellular concentration of RTK ligands, resulting from autocrine (tumor cells) or paracrine (microenvironment) production, can confer L resistance by trans-activating parallel signaling pathways (Zhang and Huang, 2011). For instance, overexpression of heregulin (HRG) or neuregulin-1 (NRG1), the main HER3 ligand, activates the EGFR-HER3-PI3K-PDK1 signaling axis, thus bypassing L-induced inhibition of HER2/EGFR. Increased HRG expression is also associated with poorer clinical outcome in HER2 + ve BC patients (Wilson et al., 2012; Xia et al., 2013). One recent study showed that P inhibits NRG1-mediated HER signaling during exposure to L, thus improving its antitumor activity and overcoming acquired resistance (Leung et al., 2015). HGF binding to MET also causes L resistance by reactivating the PI3K/AKT/mTOR pathway during HER2 pharmacological inhibition (Wilson et al., 2012; Comoglio et al., 2008).

2.2.2.2. Altered signaling by HER2-HER3 heterodimers. Different RTKs can trans-activate HER2, thus amplifying signaling pathways that result in L resistance. For instance, L-induced inhibition of HER2 induces compensatory PI3K/AKT- and FoxO3a-dependent up-regulation of HER3, thus promoting HER2-HER3 heterodimerization (Sergina et al., 2007; Garrett et al., 2011) Since P inhibits HER2-HER3/HER4 dimerization, it could be combined with L or other TKIs to revert L resistance (Table 3).

2.2.2.3. ER pathway. When exposed to L, in vitro and in vivo HER2 + ve models BC upregulate the expression of ER, progesterone receptor (PgR) and Bcl-2, which are implicated in L resistance (Xia et al., 2006; Giuliano et al., 2015). Notably, ER upregulation occurred after only 2 weeks of L-containing neoadjuvant therapy in HER2 + ve BC patients, with 18% of initially ER-ve tumors becoming ER + ve Giuliano et al., 2015). Moreover, ER inhibition with fulvestrant restored L sensitivity in preclinical HER2 + ve BC models. Therefore, reassessing ER status in L-resistant HER2 + ve BC may suggest the opportunity to combine ET to L in reviously ER-ve tumors that have become HER2 + ve.

2.2.2.4. Activation of CyclinD1- CDK 4/6 axis. As in the case of T, enhanced activation of the Cyclin D1-CDK 4/6 axis has been associated with resistance to L in HER2 + ve BC cell lines, while the CDK 4/6 inhibitor abemaciclib reverted both *de novo* and *acquired* resistance to L (Goel et al., 2016). These evidences suggest a possibly general role of CDK 4/6 activation in resistance to anti-HER2 therapies, and point to the possibility of combining anti-HER2 MoAbs/TKIs with CDK 4/6 inhibitors (Table 3).

2.2.3. Activation of pathways downstream of HER2

2.2.3.1. PI3K/AKT/mTOR pathway alterations. Aberrant activation of the PI3K-AKT-mTOR axis at different levels has been associated with resistance to L. For instance, activating mutations of *PIK3CA* (e.g., E545K and H1047R), loss of PTEN function or constitutively active AKT cause L resistance via AKT/mTOR signaling activation and FoxO3a inhibition (Eichhorn et al., 2008; Hegde et al., 2007; Gayle et al., 2012). Therefore, pharmacological inhibition of PI3K has the potential to restore sensitivity to L. Copanlisib, a pan-class I PI3K inhibitor targeting PI3K -□ and –□ isoforms, and the dual PI3K/mTOR inhibitor dactolisib (NVP-BEZ235), inhibited AKT phosphorylation and restored L sensitivity in resistant cell lines (Elster et al., 2015(Eichhorn et al., 2008; Jain et al., 2018). Activation of mTOR in both PI3K-dependent and PI3K-independent manner can similarly cause L resistance. In one preclinical study, phosphoproteomic analysis revealed increased activation of p70S6K1, a serine/threonine kinase that is a direct target of mTOR, in L-resistant cells (Vazquez-Martin et al., 2008). However, p70S6K knockdown did not fully restore L sensitivity, thus indicating a role of other mTOR targets Gayle et al., 2012). Among these candidates are inhibitors of apoptosis (IAPs) proteins, which are upregulated by mTOR in PI3K-independent manner and are overexpressed by L-resistant cells, while mTOR inhibitors prevent IAP expression and restore L sensitivity (Brady et al., 2015).

Overexpression of AXL, a transmembrane receptor containing a kinase domain similar to MET, has been detected in L-resistant HER2 + ve ER + ve BC, and is associated with poor patient prognosis (Zhang et al.,

2008). AXL binds PI3K p85 regulatory subunit, thus bypassing T- and L-mediated inhibition of PI3K/AKT/mTOR pathway. Interestingly, combining L with multi-TKIs targeting AXL restored sensitivity to anti-HER2 compounds in preclinical experiments (Liu et al., 2009).

2.2.3.2. Changes in other oncogenes/tumor suppressor genes. Aberrant activation of SRC via upregulation of the SRC family of kinases (SFKs) has also been associated with resistance to L (Roskoski, 2015; Elsberger, 2014; Rexer et al., 2011). Of note, SRC interaction with EGFR is crucial for its activation. Therefore, it is not surprising that inhibiting either SRC with saracatinib or EGFR with cetuximab reverts L resistance (Formisano et al., 2014). Protein tyrosine kinase 6 (PTK6) is a non-receptor TK highly expressed in HER2 + ve BC (Xiang et al., 2008), while PTK6 down-regulation induces apoptosis in L-resistant cells by upregulating the pro-apoptotic factor Bim1 (Park et al., 2015). Based on these evidences, PTK6 inhibition may have therapeutic potential in patients with L-resistant HER2 + ve BC.

NIK- and IKK2-binding protein (NIBP) was found to be over-expressed in tumors resistant to anti-HER2 therapies, while NIBP inhibition restores L sensitivity in resistant cells (Wetterskog et al., 2014). On the basis of the relationship between HER2, NIBP and NF-kB activation, targeting the NF-kB signaling could prove effective in combination with L.

2.3. Trastuzumab-emtansine (T-DM1)

T-DM1 is a next-generation antibody-drug conjugate (ADC) that combines the anti-HER2 effect of T with the cytotoxicity of the anti-microtubule agent DM1. To target HER2 + ve BC cells, T-DM1 needs to bind HER2 on the plasma membrane, and the HER2-T-DM1 complex needs to be internalized via receptor-mediated endocytosis. Following internalization, DM1 is released into lysosomes as a result of proteolytic degradation of the antibody part of the complex, and the Lys-MCC-DM1 metabolite of DM1 acts as a microtubule depolymerizer that inhibits cell cycle progression through mitosis (Verma et al., 2012; Krop et al., 2014). Based on results of the phase III EMILIA study, T-DM1 has been approved for the treatment of HER2 + ve mBC, and currently represents the standard second-line therapy for T-pretreated patients (Verma et al., 2012; Dieras et al., 2017). Moreover, in the recently published, phase III randomized KATHERINE trial, adjuvant T-DM1 was superior to adjuvant T in terms of invasive disease-free survival, distant recurrences and OS in patients with locally advanced HER2-positive BC and residual disease after neoadjuvant, T-containing ChT (von Minckwitz et al., 2018). While these data suggest that T-DM1 may eradicate HER2 + tumor clones that are resistant to T, both primary and acquired resistance to T-DM1 actually limit the anticancer efficacy of T-DM1 in the metastatic setting, with a median OS of less than 30 months Dieras et al., 2017).

Factors reducing T-DM1 binding to HER2, or impairing the ability of DM1 to reach a minimal intracellular concentration, can cause T-DM1 resistance (Kovtun et al., 2010). These mechanisms include: low tumor HER2 expression; impaired HER2-T-DM1 complex internalization; defective endosomal/lysosomal function that inhibits DM1 release; drug efflux pumps involved in DM1 export (Fig. 1, Table 1, Table 2). None of these mechanisms has been clinically validated yet.

2.3.1. Low HER2 expression

Low HER2 expression on tumor cell membrane prevents T-DM1 binding to its target and, consequently, its anticancer activity. In support to this hypothesis, retrospective analyses of two phase II trials (namely TDM4374 g and TDM4258 g) correlated higher HER2 protein (by IHC) and mRNA (by qPCR) levels with higher response rates during T-DM1 treatment (Krop et al., 2009; Burris et al., 2011). As in the case of T, HER2 mRNA levels correlate with benefit from T-DM1 more reliably than IHC scores, while more quantitative assessment of HER2 protein expression could further improve the ability to predict T-DM1 efficacy.

2.3.2. Impaired HER2 binding

HER2 splicing variants lacking the extracellular portion of the receptor that binds to T prevent HER2 recognition by T-DM1. Similarly to T, the p95HER2 isoform has been associated with T-DM1 resistance in preclinical studies (Arribas et al., 2011). To date, no clinical trials have evaluated the correlation between p95HER2 absolute/relative expression and T-DM1 activity.

TNF□-induced expression of MUC4 can mask the T-binding epitope of HER2; in in vitro studies, TNF□ stimulates HER2 transactivation, thus causing resistance to T-DM1 (Rivas et al., 2010). Prospective trials are currently exploring the efficacy of TNF□ inhibitors in overcoming T-DM1 resistance (Mercogliano et al., 2017).

2.3.3. Impaired cytoplasmic release/increased extrusion of DM1

T-DM1 internalization and lysosomal release of DM1 are crucial for T-DM1 to induce cell cycle G2/M arrest and mitotic catastrophe (Barok et al., 2011). Mechanisms inhibiting the release of DM1 into the cytoplasm, such as altered T-DM1 internalization through caveolae-mediated endocytosis (Sung et al., 2018), impaired lysosomal acidification and degradation of the antibody part of T-DM1 (Rios-Luci et al., 2017), or reduced export of DM1 from the lysosome into the cytoplasm through the SLC46A3 transporter (Li et al., 2018), caused acquired resistance to T-DM1 in in vitro and in vivo experiments (Fig. 1). Plasma membrane transporters of the ATP-binding cassette and solute carrier families, such as MDR1, can induce T-DM1 resistance by promoting extracellular DM1 efflux (Li et al., 2018), while MDR1 inhibitors could restore sensitivity to T-DM1.

2.3.4. Activation of signaling pathways parallel/downstream of HER2 and mitotic proteins

Overexpression of NRG1, which triggers the formation of HER2-HER3 heterodimers, has been associated with resistance to T-DM1 (Phillips et al., 2014), while inhibiting HER2-HER3 dimerization with P could restore T-DM1 sensitivity (Phillips et al., 2014). However, the P-T-DM1 combination did not prove superior to T-DM1 monotherapy in patients with HER2 + ve mBC; this suggests that NRG1 upregulation may be not clinically significant or, alternatively, that its relevance is limited to small patient subgroups (Perez et al., 2017). Similarly to other spindle poisons, DM1 inhibits tubulin polymerization and activates the spindle assembly checkpoint (SAC), which induces cyclin B1 stabilization and mitotic arrest. In one recent preclinical study, overexpression of cyclin B1 was associated with resistance to T-DM1, and quantitative assessment of cyclin B1 levels through pharmacodynamic assays could predict T-DM1 efficacy (Sabbaghi et al., 2017).

3. Dual HER2 blockade

Resistance to T-L or T-P combinations is an especially important issue in the era of standard-of-care dual HER2 blockade (Baselga et al., 2012; Swain et al., 2015; Gianni et al., 2012; Gianni et al., 2016; von Minckwitz et al., 2017). In principle, many mechanisms of resistance to T, L or T-DM1 could be common to anti-HER2 combinations; however, it is also possible that stronger upfront HER2 inhibition selects mechanisms that are qualitatively/quantitatively different from those emerging under the pressure of single HER2 blockade.

3.1. Trastuzumab plus lapatinib

Compared to single-agent L, T-L significantly prolonged the survival of patients with HER2 + ve mBC progressing after T-based therapies (Blackwell et al., 2012). In the neoadjuvant setting, T-L (plus/minus ChT or ET) increased the rate of pCR when compared to T or L alone (Carey et al., 2016; de Azambuja et al., 2014; Llombart-Cussac et al., 2017; Rimawi et al., 2013; Robidoux et al., 2013). These data support

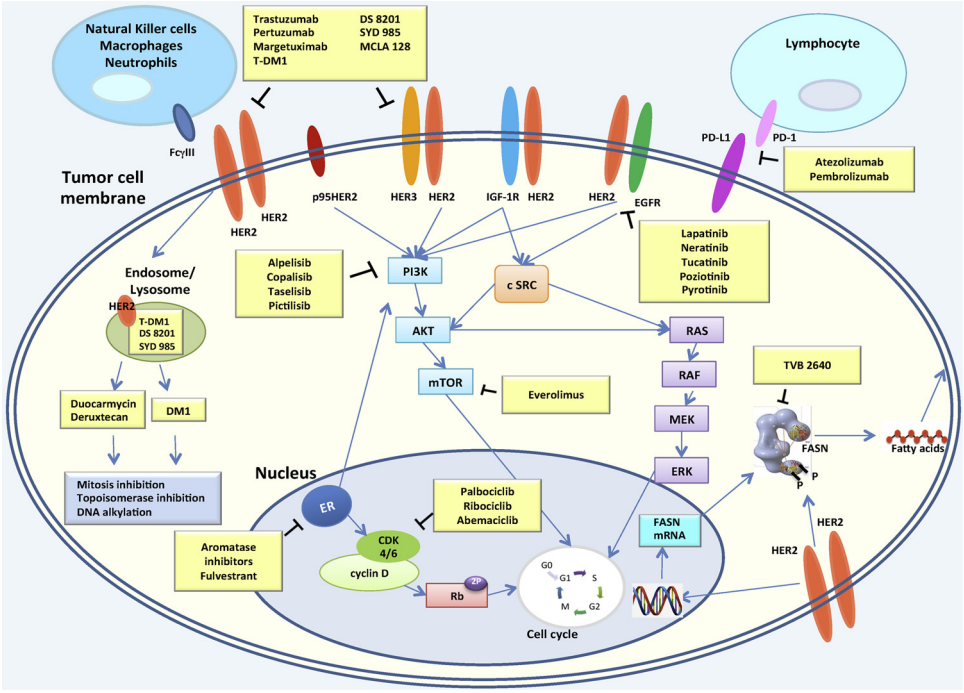

Fig. 1. Representation of the multiple intracellular signaling pathways initiated by HER2 homo- (HER2-HER2) or Heterodimerization (HER2-EGFR; HER2-HER3; HER2-IGF-1R) on the plasma membrane of HER2 + ve breast cancer cells.HER2 dimers activate the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways, thus stimulating cell growth and proliferation.SRC activation by HER2 also contributes to the stimulation of these signaling cascades.ER contributes to HER2-mediated intracellular signaling by affecting the activation status of PI3K,while it also stimulates the transcription of cyclin D1, which is implicated in the G1-S transition of the cell cycle.HER2 also induces the transcription of FASN,which in turns contributes to HER2 activation by stimulating fatty acid biosynthesis and allowing HER2 incorporation in the plasma membrane or in lipid rafts.T-DM1 and other HER2 antibody-drug conjugates (ADC) inhibit HER2 with the antibody portion of the complex, and are internalized through caveolae-mediated endocytosis; the cytotoxic part of the conjugate is then released from the lysosome into the cytoplasm, where it inhibits mitotic spindle assembly (T-DM1), topoisomerase I (DS 8201) or induces DNA damage (SYD 985).Cells of the innate immune system (Natural Killer cells, macrophages, neutrophils) can contribute to trastuzumab anticancer activity by binding trastuzumab with the FcγIII receptor,thus inducing antibody-dependent cell-mediated cytotoxicity (ADCC); new anti-HER2 antibodies, such as margentuximab, have been designed to bind FcγIII on myeloid cells more efficiently, and to produce more efficient ADCC. Conversely, the expression of PD-L1 bycancer cells inhibits the cytotoxic activity of CD8 + lymphocytes. Different compounds acting on HER2or HER2-signaling pathways, either clinically approved or under clinical investigation, are indicated in yellow rectangles. Foreachcompound, or eachclass of compounds, the molecular target is also indicated in the picture. CDK 4/6: Cyclin-dependent kinase 4/6; DM1:Derivative of Maytansine 1; EGFR: epidermal growth factor receptor; ER: estrogen receptor; FASN: fattyacid synthase;HER2:Human epidermal growth factor receptor 2;HER3:Human epidermal growth factor receptor 3;IGF-1R:insulin-like growth factor 1 receptor;mTOR:mammalian Target Of Rapamycin;PI3K:Phosphoinositide 3-kinase;PD1:Programmed death 1;PD-L1:PD1 ligand;Rb: Retinoblastoma protein (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

T-L efficacy in different clinical settings, thus highlighting the importance of finding mechanisms of primary/acquired resistance. While preclinical evidences suggest that some mechanisms of resistance to single T or L may also cause resistance to T-L (Hanker et al., 2017, 2013; Rexer et al., 2014), the frequency and clinical impact of these biological alterations is unknown, especially in the metastatic setting.

3.1.1. *Altered intracellular signaling*

One recent preclinical study found up-regulated fibroblast growth factor receptor (FGFR) signaling, as mediated by increased copy number of *FGF3/4/19* genes, in HER2 + BC xenografts with acquired resistance to T-L (Hanker et al., 2017). Pharmacological inhibition of FGFR1 in resistant neoplasms restored sensitivity to T-L, thus confirming the causative role of FGFR signaling in tumor resistance. Moreover, *FGFR1* gene amplification in primary tumors was associated with lower pCR rates in HER2 + ve BC patients receiving neoadjuvant treatment. In the same study, in vitro resistant cells had significantly lower intracellular concentrations of L, while increasing extracellular L partially restored tumor sensitivity to T-L. These data suggest that enhanced signaling through FGFR1 and reduced L uptake can synergistically contribute to T-L resistance.

Preclinical studies pointed to the importance of *PIK3CA* mutations in primary/acquired resistance to T-L (Hanker et al., 2013; Rexer et al., 2014; Rimawi et al., 2018). By acting downstream of HER2, constitutively active PIK3CA can transduce most biological signals that are usually initiated by HER2 on the plasma membrane, thus causing resistance also to dual HER2 blockade. In one analysis of 5 prospective neoadjuvant trials, *PIK3CA* mutations correlated with lower pCR rates in T-L-treated HER2 + ve BC patients (Loibl et al., 2016). However, the impact of *PIK3CA* mutations on T-L efficacy in the metastatic setting is still unknown. Based on preclinical evidences, as well as on data with T-everolimus combination (Andre et al., 2016), PIK3CA inhibitors or everolimus could delay/revert resistance to T-L in *PIK3CA*-mutated tumors. So far, no clinical trials have tested this hypothesis.

Similarly to the case of single HER2 inhibitors, specific *HER2* mutations can also cause resistance to T-L by impairing HER2 binding to L, or by disrupting the inactive conformation of HER2 kinase domain (Xu et al., 2017). The irreversible HER1/2 inhibitors afatinib and neratinib are able to target some of these mutations, and could be active in tumors with primary/acquired T-L resistance (Hyman et al., 2018).

3.1.2. *Expression/activation of ER*

As previously discussed for L monotherapy, ER can activate the PI3K/AKT/mTOR pathway, thus driving acquired resistance to T-L in HER2 + ve/ER + ve BC (Giuliano et al., 2015). Interestingly, fulvestrant restored tumor cell sensitivity to both L and T-L (Wang et al., 2011), while it was ineffective against T-resistant cells, which are still dependent on HER2-driven (and not ER-driven) signaling. In post-menopausal women with HER2 + ve/ER + ve BC, upfront treatment with aromatase inhibitors plus T-L has already demonstrated to be effective, thus confirming evidences from preclinical studies (Johnston et al., 2018; Rimawi et al., 2013).

3.2. *Trastuzumab plus pertuzumab*

T-P plus a taxane is the standard first-line treatment for patients with HER2 + ve mBC, and is FDA-approved as part of neo(adjuvant) therapy in patients with locally advanced or high-risk, early-stage disease. Uncovering mechanisms of primary/secondary resistance to dual MoAb HER2 blockade is therefore crucial to improve its efficacy in different clinical settings.

With the exception of two studies, which associated T-P resistance

to *PIK3CA*-activating mutations in transgenic HER2+ve BC mouse models (Hanker et al., 2013), or to the presence of the HER2 L755S mutation (Xu et al., 2017), the molecular bases of resistance to anti-HER2 MoAb combinations remain unknown. We can speculate that, compared to single HER2 inhibitors, mutations or overexpression of membrane TKs (e.g., HER2, HER3) are less likely to be selected by T-P, which provides wide-spectrum inhibition of HER kinases by preventing HER2 homo- and heterodimerization. Conversely, enhanced activation of signaling pathways working in parallel with HER kinases, such as FGFR1 or IGFR1, or downstream of HER2, such as PIK3CA and mTOR or, finally, aberrant expression/activation of ER, could be more easily selected as mechanisms of resistance to T-P. Albeit intriguing, these hypotheses need to be tested in preclinical and clinical studies.

4. Conclusions

Several potential mechanisms of primary/secondary resistance to anti-HER2 agents have been identified (Table 1, Table 2, Fig. 1). Most of them involve genetic or epigenetic alterations resulting in overexpression or constitutive activation of HER2/HER3/HER4 or other plasma membrane kinases (e.g. MET, FGFR1) or, alternatively, of downstream effectors. Independently from the specific mechanism, reactivation of PI3K/AKT/mTOR axis seems crucial to induce and maintain resistance to anti-HER2 therapies. In the case of T-DM1 resistance, mechanisms involving drug internalization or lysosomal function could also play a prominent role.

Quite disappointingly, despite the myriad of preclinical studies and retrospective clinical analyses published so far, the real frequency and clinical impact of the described mechanisms remain largely unclear. Indeed, most preliminary results have not been confirmed by subsequent studies. Furthermore, in the era of dual HER2 blockade, most mechanisms identified so far should be reassessed, because anti-HER2 combinations could select different alterations when compared to single HER2 blockade.

Theoretically, the majority of resistance mechanisms identified so far could be targeted by compounds that are already available, such as inhibitors of ER, PIK3CA/mTOR or FGFR1. However, the potential therapeutic advantage of combining these agents with standard HER2-targeting treatments must be weighed against the risk of causing toxicities. Moreover, the co-existence of different resistance mechanisms, as a result of intralesion or interlesion heterogeneity, may prevent the possibility to contemporaneously target all resistant tumor clones, as previously shown in other neoplasms (Pietrantonio et al., 2017). Optimizing methods to contemporaneously and reliably detect cell clones bearing different alterations associated with resistance, such as the analysis of circulating tumor DNA or circulating tumor cells, will be crucial to design treatments that are able to target the most relevant mechanisms in individual patients.

Experimental strategies that are under investigation to improve the efficacy of current anti-HER2 treatments are summarized in Table 3. They include: 1) more potent anti-HER2 MoAbs, TKIs or ADCs; 2) new anti-HER2 combinations, including new TKIs (i.e. neratinib, tucatinib, poziotinib) plus T, P or T-DM1; 3) therapies aimed at enhancing anti-tumor immune responses; 4) targeting pathways downstream of HER2, such as PI3K or CDK4/6; 5) inhibition of crucial metabolic enzymes associated with T resistance, such as FASN; 6) the design of rational treatment sequences that alternate anti-HER2 MoAbs, which deplete HER2 on tumor cell membranes, with anti-HER2 TKIs, which stimulate HER2 exposure, possibly re-sensitizing cells to anti-HER2 MoAbs (Vici et al., 2017; Scaltriti et al., 2009; Fabi et al., 2017).

Conflict of interest statement

Claudio Vernieri has no conflict of interest to declare
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