NANOPARTICLE BASED RNAI THERAPEUTIC TO OVERCOME INTRINSIC AND ACQUIRED RESISTANCE IN HER2+ BREAST CANCER

By

Shenda Gu

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Shenda Gu

has been approved

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Dissertation Advisor: Wassana Yantasee, PhD, MBA

_____

Committee Chair: Paul Spellman, PhD

_____

Committee Member: Jim Korkola, PhD

_____

Committee Member: Jeffrey Tyner, PhD

_____

Committee Member: Tania Vu, PhD

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ABSTRACT

The HER2 receptor tyrosine kinase is overexpressed in approximately 20% of all breast cancers and is a poor prognostic indicator. Hyperactive HER2 signaling leads to aggressive tumor growth, metastasis, and resistance to traditional chemotherapy. The use of HER2 targeted therapies such as trastuzumab and lapatinib has dramatically prolonged survival compared to chemotherapy treatment alone. Unfortunately, a significant number of patients do not respond to these therapies while others will eventually relapse, prompting the need for better alternatives. We hypothesized that the ablation of HER2 protein by RNA interference would be a more effective and durable therapeutic approach. As such, I systematically screened and identified an optimal HER2 siRNA from a large pool of candidates and demonstrated that it could overcome intrinsic and acquired resistance to trastuzumab and lapatinib *in vitro*. Next, we validated the *in vivo* efficacy of the HER2 siRNA in a mouse xenograft model of trastuzumab resistant HER2+ breast cancer using a HER2 targeted nanoparticle platform recently developed in our lab. Finally, I showed that long-term treatment with HER2 siRNA on our nanoparticles did not lead to acquired resistance, in contrast to trastuzumab and lapatinib. In summary, our HER2 siRNA nanotherapeutic is more effective than current HER2 targeted therapies and therefore warrants further investigation for clinical applications.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADAM10	a disintegrin and metalloproteinase domain-containing protein 10
ADCC	antibody-dependent cell-mediated cytotoxicity
AGO2	argonaute 2
AKT	protein kinase B
AON	antisense oligonucleotide
ApoE	apolipoprotein E
ATP	adenosine triphosphate
BCL2	B-cell lymphoma 2
BLAST	basic local alignment search tool
CD24	cluster of differentiation 24
CD44	cluster of differentiation 44
CDK2	cyclin-dependent kinase 2,
CHC	caveolin heavy chain
CTF	c-terminal fragment
CYP3A4	cytochrome P450 3A4
DsiRNA	dicer substrate RNA
ECD	extracellular domain
EGFR	epidermal growth factor
EMA	European Medicines Agency
EMT	epithelial-mesenchymal transition
EphA2	ephrin type-A receptor 2

ER	estrogen receptor
ERBB2	receptor tyrosine-protein kinase erbB-2
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
Fc	fragment, crystallizable
FDA	Food and Drug Administration
FGFR	fibroblast growth factor receptor
GFP	green fluorescent protein
GRB2	growth factor receptor-bound protein 2
HER	human epidermal growth factor receptor
HER2	human epidermal growth factor receptor 2
HER3	human epidermal growth factor receptor 3
HER4	human epidermal growth factor receptor 4
HGF	hepatocyte growth factor
IGFR	insulin-like growth factor 1 receptor
LDLR	low-density lipoprotein receptor
MAPK	mitogen-activated protein kinase
MCT	monocarboxylate transporter
MDR	multidrug resistance
MET	tyrosine-protein kinase Met
miRNA	microRNA
MPS	mononuclear phagocyte system
MRI	magnetic resonance imaging

MSNP	mesoporous silica nanoparticle
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
MUC1	mucin 1
MUC4	mucin 4
NCL	nanotechnology characterization lab
p27kip1	cyclin-dependent kinase inhibitor 1B
P450	cytochromes P450
PAMAM	polyamidoamine
PDK1	phosphatidylinositol-dependent kinase 1
PEG	polyethylene glycol
PEI	polyethylenimine
PH	plekstrin homology
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PLA	polylactic acid
PLGA	polylactic acid-co-glycolic acid
PTEN	phosphatase and tensin homolog
PTK6	tyrosine-protein kinase 6

RAF	RAF proto-oncogene serine/threonine-protein kinase
RAS	RAS GTPase
RefSeq	reference sequence
RES	reticular endothelial system
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNAseq	RNA sequencing
RPPA	reverse phase protein array
SD	standard deviation
SEM	standard error of the mean
SH	src homology
SHC1	SHC-transforming protein 1
shRNA	small hairpin RNA
siRNA	small interfering RNA
SLC1A5	neutral amino acid transporter B
SLN	solid lipid nanoparticles
SNALP	stable nucleic acid lipid nanoparticles
SOS	son of sevenless
STAT3	signal transducer and activator of transcription 3
TCGA	the cancer genome atlas
T-DM1	trastuzumab emtansine
TIC	tumor initiating cell
UTR	untranslated region

CHAPTER 1

INTRODUCTION AND BACKGROUND

The HER2 Oncogene

The human epidermal growth factor receptor 2 (HER2) is a 185 kD transmembrane receptor tyrosine kinase of the HER family (1-3), which also includes EGFR, HER3 and HER4 (4). While HER2 is expressed in a variety of normal tissues derived from the ectoderm and mesoderm, overexpression of this protein leads to tumorigenic transformation (5). It is estimated that on average, 20% of all breast cancer falls into the HER2+ subtype (6). HER2 is also overexpressed in other types of cancer such as ovarian, gastric, colorectal, bladder, colon, skin and lung (7). *ERBB2*, the gene encoding the HER2 protein, is located on chromosome 17 (8), which is amplified in HER2+ breast cancer. Clinically, the HER2+ classification is determined via immunohistochemistry staining or in-situ hybridization, but does not always correspond to the HER2-enriched intrinsic subtypes using the PAM50 signature (9).

All 4 receptors of the HER family share sequence and structural homology (10). Like most receptor tyrosine kinases, HER receptors have extracellular domains for ligand binding and receptor dimerization, an intracellular protein kinase domain (11) and a tyrosine-rich c-terminal tail as site for autophosphorylation (12). The extracellular region of HER receptors is composed of four domains. Domains I and III are leucine-rich and function in ligand binding

(13). Domains II and IV are cysteine-rich and are responsible for receptor dimerization (14). See Figure 1.1.

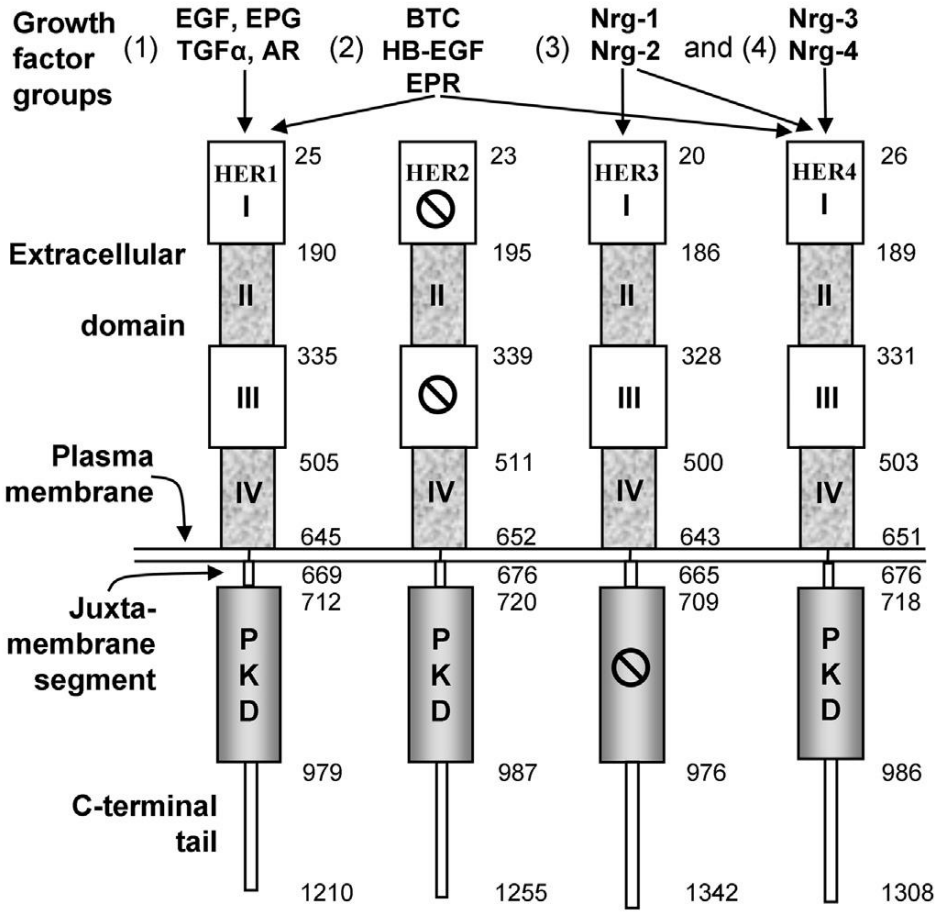

Figure 1.1 Structures and ligands of HER family receptors. HER receptors share sequence and structural homology. Domains I and III function in ligand binding. Domains II and IV serve as dimerization arms. The cytoplasmic tail is the site of autophosphorylation. HER2 has no endogenous ligand while HER3 has no kinase activity. Reprinted with permission from (4).

Activation of HER receptors normally requires binding of a ligand, which induces a confirmation change that facilitates the dimerization of the receptor (15) and subsequent phosphorylation of the cytoplasmic tail (12). The dimerization of HER receptors is asymmetric, in which the C-terminal of the kinase domain of one receptor binds to the N-terminal of the kinase domain of the other to achieve an active conformation (16). HER receptors have numerous ligands, some of which bind to more than one member of the family (17). HER2 can form heterodimers with any other HER receptors (18) or simply homodimerize (10). While HER2 does not appear to have an endogenous ligand, it is always in an active-like conformation (19) and preferentially heterodimerizes with HER3, which has a non-functional kinase domain (20). The HER2/HER3 heterodimer was shown to be a potent oncogenic driver in HER2+ breast cancer (21) and associated with poor survival (22).

HER2 signaling pathway

Signaling downstream of HER2 is primarily mediated through the PI3K-AKT and the RAS-MAPK pathways, which regulate the cell's survival and proliferation. PI3K is a heterodimer made up of a p85 regulatory unit and a p110 catalytic unit. When the cytoplasmic tail of HER2 is phosphorylated, p85 is recruited to the cell membrane and binds the phosphorylated tyrosine residues (23). The catalytic unit then in turn converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (23). AKT and phosphatidylinositol-dependent kinase 1 (PDK1) are recruited to the membrane and bind to PIP3 via

their plekstrin homology (PH) domains (24). PDK1 is active at this point and activates AKT by phosphorylating threonine residue 308 (24). Full activation of AKT also requires the phosphorylation of serine residue 473, which is carried out by the mammalian target of rapamycin complex 2 (mTORC2) (24). The phosphatase and tensin homolog (PTEN) dephosphorylates PIP3 back to PIP2 (24). See Figure 1.2.

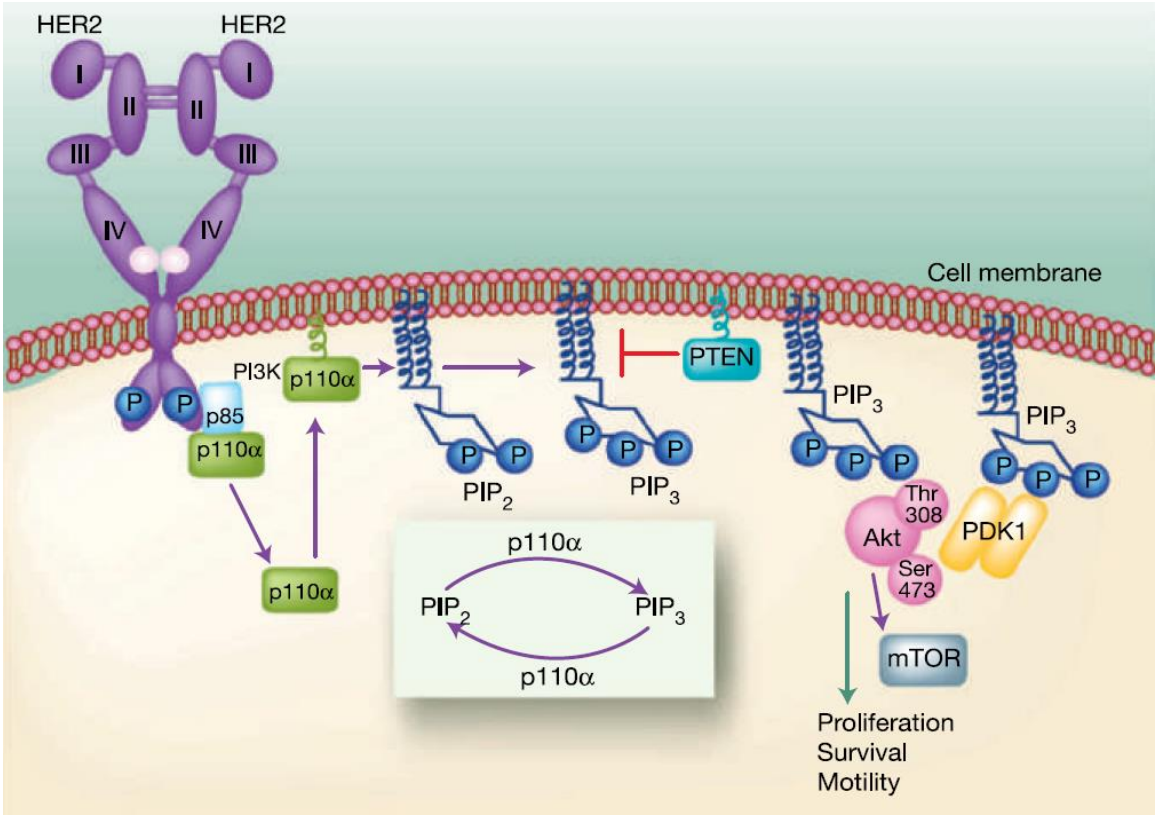

Figure 1.2 The PI3K-AKT signaling pathway. Upon receptor activation, the regulatory unit of PI3K binds to the phosphorylated tail of HER2. The catalytic unit phosphorylates PIP2 to PIP3. PDK1 is recruited to the membrane and it activates AKT by phosphorylating threonine 308. Reprinted with permission from (25).

In the MAPK pathway, the adaptor proteins GRB2 and SHC1 bind to the phosphorylated tyrosine residues on HER2. GRB2 can also bind indirectly via SHC1 first, both of which contain the Src homology (SH) domain (26). The guanine nucleotide exchange factor SOS is then recruited, which in turn promotes the release of GDP from RAS (26). RAS is activated after binding to a free GTP from the cytoplasm, phosphorylates RAF and subsequently MEK and MAPK (26). See Figure 1.3.

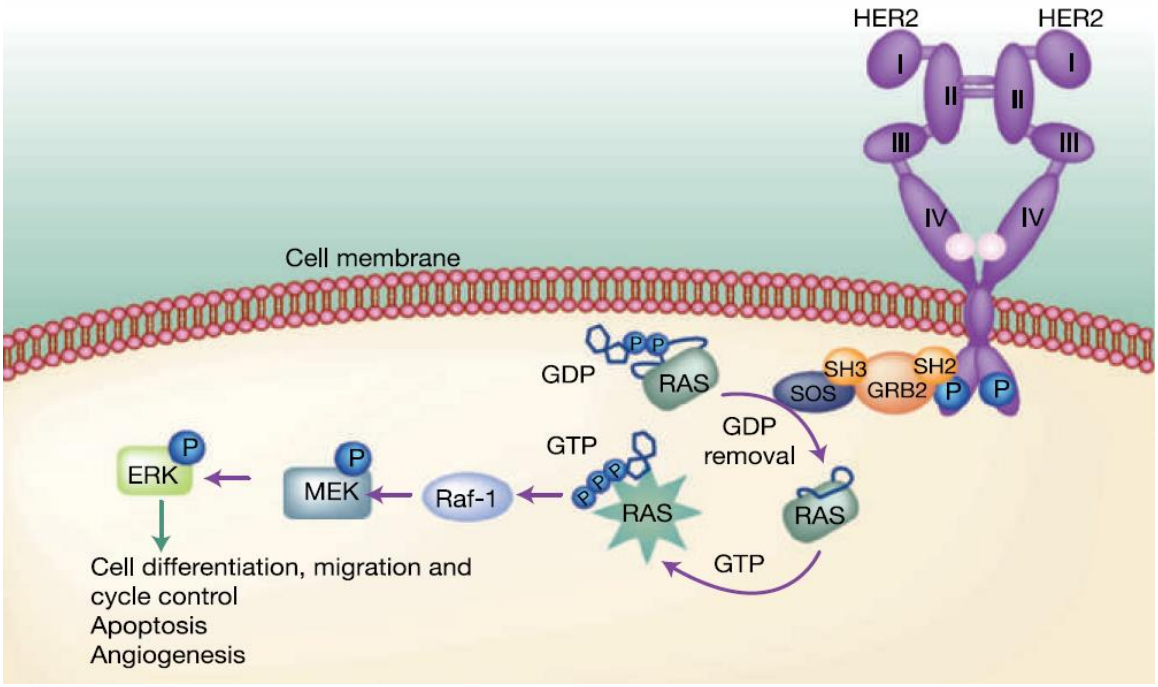

Figure 1.3 The RAS-MAPK signaling pathway. Activated HER2 recruits the adaptor proteins GRB2 and SOS, which activate RAS. The signaling cascade continues with RAF, MEK and ultimately ERK (MAPK). Reprinted with permission from (25).

HER2 targeted therapies

Current FDA approved therapeutics targeting HER2 include the monoclonal antibodies trastuzumab and pertuzumab, the drug-antibody conjugate T-DM1, and the small molecule tyrosine kinase inhibitors lapatinib and neratinib. These agents are often used in combination with traditional chemotherapy. Figure 1.4 illustrates the sites of action of HER2 targeted therapies.

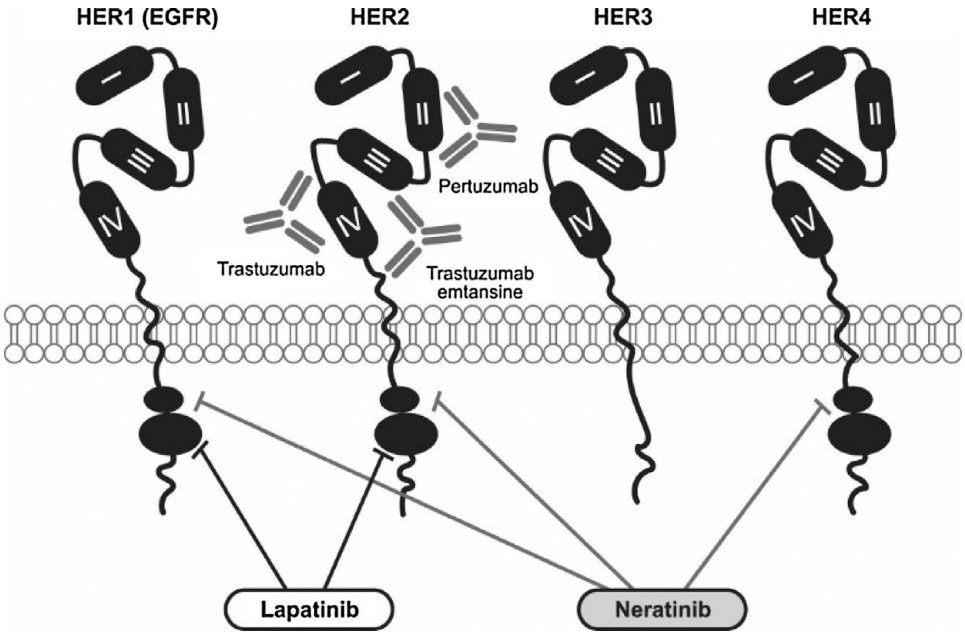

Figure 1.4 Sites of action of HER2 targeted therapeutics. Trastuzumab binds domain IV of HER2 and blocks receptor homodimerization. Pertuzumab binds domain II and blocks receptor heterodimerization. In addition to the effect of trastuzumab, trastuzumab emtansine (T-DM1) inhibits microtubule assembly. Lapatinib and neratinib bind the ATP pocket in the kinase domain and block the kinase activity. Reprinted with permission from (27).

Trastuzumab

Trastuzumab (4D5, trade name Herceptin®) is the first humanized monoclonal antibody targeting HER2 developed in the early 90's (28). The addition of trastuzumab to chemotherapy led to significant improvement in overall survival in patients with HER2+ metastatic breast cancer (29). Trastuzumab in combination with chemotherapy was also investigated in the adjuvant settings post-surgery and showed positive results (30). It is currently approved for both scenarios (31). However, cardiac toxicity is a major adverse effect, especially when given with doxorubicin (29, 30, 32, 33).

Trastuzumab works mainly by blocking HER2 homodimerization and by inducing antibody-dependent cell-mediated cytotoxicity (ADCC). The binding of trastuzumab to the extracellular domain IV of HER2 hinders dimerization with another HER2 protein (34). *In vitro* studies suggest that reduced HER2 signaling leads to stabilization of the cyclin dependent kinase inhibitor p27kip1, a target of AKT (35). By forming complex with CDK2, p27kip1 induces cell cycle arrest in the G1 phase (36). Since trastuzumab is a humanized antibody, it has a human Fc region that can be recognized by Fcγ receptors on natural killer cells and therefore induce an innate immune response (37). This mechanism is supported by studies showing that F(ab')2 fragments of anti-HER2 antibodies did not illicit the same level of anti-tumor activity (38) and reduced trastuzumab efficacy in Fcγ receptor deficient mice (37). Further evidence comes from genotyping of patients who received trastuzumab, where those with the FcγRIIIa-158 V/V or FcγRIIa-131 H/H genotype had better objective response rate or progression-free survival (39).

Pertuzumab

Pertuzumab (2C4, trade name Perjeta®) is the second monoclonal antibody targeting HER2 developed by Genentech (40). Following poor results from a phase 2 clinical trial as a monotherapy, it was subsequently investigated in combination with trastuzumab (41). In the CLEOPATRA phase 3 trial, the addition of pertuzumab to trastuzumab plus docetaxel significantly improved overall survival in patients with HER2+ metastatic breast cancer (42). After receiving approval for treating metastatic breast cancer, the combination of pertuzumab, trastuzumab and docetaxel also received accelerated approval for treating locally advanced and early stage breast cancer (43).

The mechanism of action of pertuzumab is similar to that of trastuzumab. However, pertuzumab binds to the extracellular domain II of HER2 and prevents heterodimerization of HER2 with other members of HER family (44). The combination of trastuzumab and pertuzumab can therefore provide a more complete blockade of HER2 activation.

T-DM1

T-DM1 or trastuzumab emtansine (trade name Kadcyla®) is an antibody-drug conjugate composed of trastuzumab attached to emtansine, a cytotoxic agent that blocks the assembly of tubulin (45). T-DM1 was approved following positive results of the EMILIA phase 3 trial, which investigated the efficacy of T-DM1 versus lapatinib plus capecitabine in patients with metastatic cancer who have relapsed on trastuzumab plus chemotherapy (46, 47). In the TH3RESA phase 3 trial, T-DM1

was effective in treating patients who have progressed on both trastuzumab and lapatinib (48). However, in the MARIANNE phase 3 trial, T-DM1 was not superior to trastuzumab plus a taxane for first-line treatment of locally advanced and metastatic HER2+ breast cancer, and therefore remains as second-line treatment (49).

Lapatinib

Lapatinib (GW572016, trade name Tykerb®) is a reversible dual EGFR and HER2 inhibitor that is orally active (50). Although lapatinib has anti-proliferative activity as a single agent (51), it was investigated for use with capecitabine in a phase 3 clinical trial and showed greater efficacy than capecitabine alone for patients who have received prior trastuzumab and chemotherapy (52). Similar results were obtained from trials studying the benefit of lapatinib when combined with a taxane versus taxane alone (53). As a first-line treatment for advanced or metastatic HER2+ breast cancer, the combination of lapatinib and a taxane was not superior over trastuzumab plus a taxane in the NCIC CTG MA.31 phase 3 trial (54). Since T-DM1 has greater efficacy over lapatinib plus capecitabine (47), lapatinib plus a chemotherapy is now used as third-line treatment. In the neoadjuvant setting, there was no significant difference in overall survival among the lapatinib, trastuzumab, and lapatinib plus trastuzumab groups while the lapatinib containing groups had more adverse events (55).

Lapatinib competes with ATP in the ATP binding site of the protein kinase. Binding of lapatinib blocks the kinase activity of HER2, leading to reduced signaling

(56). Consistent with the results of clinical trials, an *in vitro* study showed that lapatinib could overcome HER2+ cells that have become resistant to trastuzumab (57, 58). In addition to blocking HER2 signaling, lapatinib also appears to induce HER2 accumulation on the cell membrane, thereby enhancing the trastuzumab induced ADCC (59, 60).

Neratinib

Neratinib (HKI-272, trade name Nerlynx™) is an irreversible pan-HER inhibitor that is also orally active but has much higher potency than lapatinib (61). It is currently approved for extending adjuvant therapy in patients with early stage disease who have received adjuvant trastuzumab therapy within one year or less (62). The phase 3 ExteNET trial showed that neratinib as a single agent gives a better 2-year invasive-disease free survival rate in these patients than the placebo (63). Neratinib is currently being investigated for use in the neoadjuvant settings (64) as well as in advanced HER2+ breast cancer (65).

In vitro studies have shown that neratinib can partially overcome resistance to lapatinib and trastuzumab (66). Cells that were selected to become resistant to neratinib were also cross resistant to other HER2 targeted therapies (67). The same study also showed that higher activity of the P450 metabolic enzyme CYP3A4 contributes to neratinib resistance, as chemical inhibition of this enzyme in the resistant cells partially restored their sensitivity to neratinib. In contrast to lapatinib, neratinib induces surface downregulation of HER2 by dissociating HSP90, leading to ubiquitination and subsequent degradation of the receptor (68).

Other HER2 targeting agents and regimens

A few other tyrosine kinase inhibitors were being investigated in the preclinical and clinical stages. Afatinib (BIBW2992, Gilotrif®) is an irreversible inhibitor of EGFR and HER2 (69) that is currently approved for treating metastatic non-small cell lung cancer with exon 19 deletion or exon 21 L858R substitution in the EGFR receptor (70). However, in the LUX-Breast 1 phase 3 clinical trial, afatinib plus vinorelbine did not show superiority over trastuzumab plus vinorelbine in patients who had progressed on trastuzumab (71). After poor results from the concurrent, LUX-Breast 3 trial, Boehringer Ingelheim has abandoned further investigation for treating HER2+ breast cancer (72). Sapitinib (AZD8931) is a reversible inhibitor of EGFR, HER2 and HER3 (73) but has never been investigated in the clinics for HER2+ breast cancer. In a phase 1 trial, tucatinib (ONT-380) had anti-tumor activity comparable to lapatinib or neratinib but with lower adverse events in patients with advanced or metastatic breast cancer (74). A phase 2 trial investigating the combination of tucatinib with trastuzumab plus capecitabine is ongoing (75).

Novotny and colleagues have developed kinase inhibitors that specifically target the active conformation of HER2/HER3 dimers. Kinase inhibitors such as lapatinib can only bind to the kinase domain of HER2 when it is in the inactive form. These active state specific inhibitors were therefore able to overcome resistance due to ligand-induced activation of HER2/HER3 dimers (76).

As the patent protection is ending for trastuzumab in 2019 in the U.S. and has already expired in Europe since 2014, biosimilars are beginning to appear on

the market. They are equivalent of existing biologics and are likely to drive treatment cost down through competition. The FDA has recently approved Mylan's Trastuzumab-dkst (MYL-1401O, trade name Ogivri). The European Medicine Agency (EMA) has also approved Ontruzant, a trastuzumab biosimilar developed by Samsung Bioepsis.

A HER2 targeted liposomal form of doxorubicin MM-302 (77) made it to the phase 2 HERMIONE clinical trial (78). However, the trial was stopped midway after interim results showed no benefit over trastuzumab plus traditional chemotherapy in patients with locally advanced or metastatic HER2+ breast cancer who had previously received trastuzumab, pertuzumab or T-DM1, but not anthracycline (79).

A HER2 peptide (E75) based vaccine, Nelipepimut-S (NeuVax™, Sellas Life Sciences), met its safety and efficacy endpoints in phase 2 clinical trials (80) but the phase 3 PRESENT trial was stopped after analysis of interim results indicated unlikeliness to achieve statistical significance (81). However, two phase 2 trials investigating the combination of NeuVax and trastuzumab are still ongoing (82, 83).

Some of the mechanisms of resistance to be discussed in the next section have prompted clinical trials investigating combination therapy by blocking HER2 and downstream signaling protein or other surface receptors. In the Bolero 1 phase 3 trial, the addition of the mTOR inhibitor everolimus to trastuzumab plus paclitaxel did not improve progression free survival (84). The PI3K inhibitor buparlisib, when combined with trastuzumab or lapatinib, have met safety endpoint in their respective phase 1b trials for treating advanced HER2+ positive cancer resistant

to trastuzumab (85, 86). However, in the neoadjuvant settings, buparlisib was not well tolerated when used in combination with trastuzumab plus paclitaxel in the NeoPHOEBE phase 2 trial (87). Another PI3K inhibitor, pilaralisib, also demonstrated promising safety profile and anti-tumor activity when combined with trastuzumab or trastuzumab plus paclitaxel in a phase 1/2 trial (88).

Mechanisms of resistance to HER2 targeted therapies

Despite bringing improvements over chemotherapy alone, HER2 targeted therapies are not always effective. Results from clinical trials showed that approximately 50% of patients do not respond to trastuzumab and most will relapse after a year and half of therapy (29). Similar response profiles were observed for lapatinib (52) and T-DM1 (47). Some cancer cells are therefore *de novo* (intrinsic) resistant prior to treatment while others acquire resistance during treatment.

Several mechanisms of resistance have been proposed and studied. As an adaptive response to HER2 targeted therapy, cancer cells can upregulate the expression of other HER family receptors to compensate for the inhibition of HER2 (76, 89-93). Resistant cells can also increase autocrine signaling by secreting their own HER receptor ligands (89, 94). There are numerous reports of receptor cross talk and compensation through alternative signaling pathways, often involving HER2 binding to a non-canonical signaling partner (95-104). Upregulation of certain membrane glycoproteins can sterically block the binding of trastuzumab and enhance the phosphorylation of HER2 (105-111). Dysregulation of

downstream signaling kinases and cell cycle regulators can affect sensitivity to HER2 targeted therapy (112-114). In addition, certain HER2 mutations and splice variants lack an extra cellular domain for therapeutic antibody to bind, have higher affinity for ATP than kinase inhibitors, or promote constitutive receptor activation (115-124). Finally, attenuation of ADCC can compromise the therapeutic effect of trastuzumab and pertuzumab (39).

Upregulation and signaling through other HER family receptors

Upregulation of EGFR can offset the therapeutic effect of trastuzumab. Cells isolated from an in vivo xenograft model of trastuzumab resistance showed high level of EGFR expression and EGFR/HER2 heterodimers. These cells also secrete HER receptor ligands such as EGF, TGFα and neuregulin. As expected, the resistance to trastuzumab could be managed by using the EGFR inhibitors erlotinib and gefitinib or the dual EGFR/HER2 inhibitor lapatinib (89).

HER3 plays a major role in mediating refractory response to lapatinib. Upon lapatinib treatment, the expression and activity of HER3 is often upregulated via a negative feedback loop through AKT (90). Upregulation of HER3 can be achieved via several mechanisms, including an increase in transcription and translation, higher mRNA stability, a decrease in dephosphorylation of the receptor, and increased membrane localization (91). The binding of neuregulin to HER3 can induce a confirmation change that blocks lapatinib from accessing the ATP binding pocket (76). In addition, nuclear localization of HER2/HER3 dimers also contributes to trastuzumab resistance (125). In their study, Russo and colleagues

have shown that upon neuregulin stimulation, HER2/HER3 dimers are internalized and form a transcriptional complex with signal transducer and activator of transcription 3 (STAT3) at the cyclin D1 promoter. The expression of a nuclear localization signal deficient variant of HER2 abolished neuregulin dependent growth of trastuzumab resistant cells in vitro in and tumors in vivo (125).

In an *in vitro* model of trastuzumab resistance, concurrent neuregulin and trastuzumab treatment induced upregulation of HER4 and nuclear translocation of $HER4_{80kD}$ (93). $HER4_{80kD}$ is a cytoplasmic fragment generated from the cleavage of the transmembrane domain by gamma-secretase (126). Knocking down HER4 or inhibiting gamma-secretase restored response to trastuzumab (93). Neratinib was also able to block HER4 upregulation and nuclear translocation (93). Analysis of patient samples showed that nuclear HER4 correlates with poor survival and predicts reduced response to trastuzumab (93). Similar results were obtained in a separate study, which showed that lapatinib and trastuzumab resistant cells were sensitive to HER4 silencing (92). However, the in vivo model using MMTV-neu mice from the same study showed membrane localization of HER4 upon lapatinib treatment, rather than nuclear translocation. In addition, lapatinib resistance could not be induced by simply overexpressing a constitutively active form of HER4, suggesting that there are additional mechanisms involved (92).

Receptor cross-talk and alternative signaling pathways

In hormone receptor positive HER2+ breast cancer, the initial resiliency of cancer cells towards trastuzumab and lapatinib can be mediated through estrogen

receptor (ER) signaling. *In vitro* and in vivo xenograft studies showed that in the early stage of resistance, high ER expression and signaling promoted survival via upregulation of BCL2, which can be managed via estrogen deprivation and fulvestrant treatment or inhibition of BCL2 (95). However, in the later stage of resistance where the cells have been exposed to lapatinib longer than 6 months, HER2 signaling was reactivated via secretion of HER ligands (94).

Signaling through insulin-like growth factor 1 receptor (IGFR) can also attenuate the activity of trastuzumab. MCF7/HER2-18 cells, which express both IGFR and HER2, did not respond to trastuzumab in the presence of insulin-like growth factor 1 (IGF) or fetal bovine serum *in vitro* (99). Overexpressing IGFR in the otherwise IGFR- but HER2+ SKBR3 cells also rendered these cells insensitive to trastuzumab (99). Dual inhibition of HER2 and IGFR was therefore found to be synergistic (98). In a subsequent study, IGFR signaling downregulated p27kip1 via activation of AKT pathway, which blocked the cell cycle arrest effect of trastuzumab (97). IGFR was later shown to be heterodimerizing with HER2, which can be partially blocked by pertuzumab and more fully by an anti-IGFR antibody (96). However, unlike the IGFR inhibitor I-OMe-AG538, disrupting receptor heterodimerization did not reduce phosphorylation of HER2 (96).

An increase in fibroblast growth factor receptor (FGFR) signaling was observed in xenograft models resistant to lapatinib and trastuzumab (100). In the resistant tumors, the intratumoral concentration of lapatinib was significantly lower than in non-resistant tumors (100). The resistant tumors also have an increase in the copy number of fibroblast growth factors (FGF) 3, 4 and 19 (100). Stimulation

of *in vitro* cultured cells with FGF4 was able to sustain phosphorylation of HER2 and downstream signaling molecules in the presence of lapatinib (100). Inhibition of FGFR and HER2 was synergistic and could restore sensitivity to lapatinib and trastuzumab in the resistant tumors in vivo (100). Analysis of clinical samples from the FinHER and NeoALLTO trials showed correlation between high FGFR1 expression and reduced response to trastuzumab in the adjuvant and neoadjuvant settings, respectively (100). A separate study also implicated FGFR2 in promoting resistance to trastuzumab (101). High FGFR2 expression was found in human and transgenic mouse tumor tissues overexpressing HER2 (101). *In vitro* stimulation with the FGFR2 ligand FGF7 led to an increase in membrane HER2 localization and receptor shedding to produce p95HER2, a truncated and more oncogenic form of HER2 (101).

High expression of MET, the receptor tyrosine kinase for hepatocyte growth factor (HGF), was detected in a number of HER2+ cell lines and tumor tissues (102). *In vitro* study in SKBR3 and T474 cells showed that HGF stimulation promoted cancer cell growth and trastuzumab resistance (102). MET expression was rapidly upregulated in cells treated with trastuzumab (102). Conversely, inhibition of MET enhanced the growth inhibitory effect of trastuzumab (102). Evidence from clinical samples supported the *in vitro* finding, showing upregulation of MET in trastuzumab resistant tumors (102). Paulson and colleagues found a similar pattern of HER2 and MET coexpression in a subset of HER2+ breast tumor (103). However, in their *in vitro* study using HCC1954 cells, HER2 and MET

compensated for each other in term of phosphorylation when either receptor was knocked down using shRNA.

The expression and activation of another receptor tyrosine kinase, AXL, was higher in an *in vitro* model of lapatinib resistance based on BT474 cells (104). Treatment with the multi-kinase inhibitor foretinib was able to restore sensitivity to lapatinib. Because foretinib also targets MET and vascular endothelial growth factor receptor (VEGFR), MET or VEGFR specific inhibitors were used as control and did not reverse lapatinib resistance. Interestingly, AXL appeared to be regulated by ER as its expression was downregulated by estrogen deprivation and fulvestrant treatment (104).

Mucins are a family of highly glycosylated membrane associated proteins (127). In a rat model of breast cancer, Muc4 formed complexes with Erbb2. Ectopic expression of rat Muc4 in a human melanoma cell line increased HER2 signaling following neuregulin stimulation (110). When expressed in the human breast cancer cell line MCF7, Muc4 could sterically hinder trastuzumab binding (109). A separate group performed a similar study using MCF7 and T47D cell lines and demonstrated that the potentiation of neuregulin activity was mediated through accumulation of HER2/HER3 homodimers at the cell membrane (107). Similar to findings from studies of rat Muc4, high endogenous expression of MUC4 on the surface of JIMT1 cells could partially block trastuzumab binding (108). The binding of the smaller Fab fragment of trastuzumab and pertuzumab, which targets a region on HER2 that is more distal from the cell membrane, was less affected. As expected, knocking down MUC4 increased the binding of trastuzumab (108). A

more recent study working with an in vivo xenograft model of endocrine, lapatinib and trastuzumab resistance also reported higher MUC4 expression in the resected tumor tissues (106).

Another member of the mucin family, MUC1, has also been shown to contribute to trastuzumab resistance. MUC1 undergoes autocleavage to produce an N-terminal MUC1-N and a C-terminal MUC1-C. Shedding of MUC1-N leaves MUC1-C at the cell membrane to form complexes with receptor tyrosine kinases (128). High levels of MUC1-C were measured in BT474 cells that were selected to become resistant to trastuzumab (111). The Fab fragment of an anti-MUC1-C antibody was able to reverse trastuzumab resistance in these acquired resistant BT474 cells, as well as in T47D and ZR75-30 cells that have intrinsic resistance (111). Raina and colleagues reported similar findings in their version of acquired resistant BT474 and SKBR3 cells (105). They also showed that MUC1-C formed complexes with HER2 in the resistant cells. Inhibition of MUC1-C by siRNA or inhibitor blocked the phosphorylation of HER2 and HER3 and restored trastuzumab sensitivity in the resistant cells (105).

PTEN loss and PIK3CA mutation

The loss of PTEN and PIK3CA mutation can negatively affect the efficacy of trastuzumab. A study showed that the anti-proliferative effect of trastuzumab is mediated through the upregulation of PTEN *in vitro* and *in vivo*, whereas silencing of PTEN led to reduced trastuzumab efficacy, which can be restored by inhibiting PI3K (112). In HER2+ cell lines, low PTEN and/or PIK3CA mutation conferred

resistance to trastuzumab but not always to lapatinib (129). Cells with PIK3CA mutations that are resistant to lapatinib are vulnerable to PI3K (130, 131) or AKT inhibition (132). Analysis of clinical samples, however, only supported PIK3CA mutation or a combination of PIK3CA and PTEN loss as a predictor of trastuzumab response in most neoadjuvant and metastatic settings (133-140). In the adjuvant setting, neither PI3KCA mutation nor PTEN loss were found to be associated with trastuzumab efficacy (141-143). Moasser and Krop have argued that observations from the neoadjuvant and metastatic settings were simply reduced response to chemotherapy rather than trastuzumab resistance, since none of studies included a chemotherapy alone control to properly assess the true efficacy of trastuzumab (144).

Cell cycle dysregulation

Overexpression of cyclin E has been associated with trastuzumab resistance. Clinical samples showed a positive correlation between cyclin E expression and reduced response to trastuzumab (114). The same study also showed that BT474 cells with acquired resistance to trastuzumab expressed elevated level of cyclin E *in vitro*. The binding of cyclin E to CDK2 is required for progression through the G1 phase of cell cycle (145). Therefore, the inhibition of CDK2 markedly reduced growth of resistant cells *in vitro* and *in vivo* (114). One mechanism of action of trastuzumab is the upregulation the CDK2 inhibitor p27kip1 (35, 36). Decreased level of p27kip1 and high CDK2 activity have been reported

in SKBR3 cells that were made resistant to trastuzumab (113), which is likely the result of signaling from other receptors such as IGFR as discussed earlier.

Mutations

A number of activating mutations in the kinase domain have been reported and characterized. According to patient tumor data from the Cancer Genome Atlas (TCGA), the overall rate of HER2 mutation is only about 3% in breast cancer (66). Mutations have also been found in cases where HER2 is not amplified (146). Compared to wild type HER2, mutated HER2 is associated with reduced overall survival in invasive breast carcinomas, regardless of HER2 amplification status (147). The kinase domain mutations V777L, D769H and V842I have greater signaling activity *in vitro* and xenograft tumor growth potential in vivo (146). Another kinase domain mutation, L755S, is associated with lapatinib resistance (66, 148). Several other HER2 mutations conferring lapatinib resistance have been reported in lung, ovarian, gastric, colon and liver cancers but not in breast (148, 149).

p95HER2

The full length 185kD HER2 can undergo proteolytic cleavage by the metalloprotease ADAM10 to produce a soluble 110 kDa extra cellular domain (ECD) fragment and a transmembrane 95 kDa c-terminal fragment (CTF), termed p95HER2 (150). P95HER2 can also be generated from the alternative initiation of translation at methionine 611, also referred to as 611-CTF (151). The

transmembrane fragment readily forms homodimers and is constitutively active, and is therefore highly oncogenic and promotes metastasis (152). Clinically, patients with high levels of serum ECD and p95HER2 have worse outcomes with more frequent metastasis to the lymph nodes (115, 116). While an early *in vitro* study suggests that trastuzumab can block shedding of soluble HER2 (153), more recent clinical data showed that patients with p95HER2 have reduced response to trastuzumab (117). Without an extra cellular domain, trastuzumab has no epitope to bind. However, because p95HER2 still relies on its kinase activity, kinase inhibitors such as lapatinib remain effective at inhibiting this variant (117, 154).

Δ16HER2

An alternative splice form of HER2 called Δ16HER2 where exon 16 is skipped was associated with trastuzumab resistance (120). This in-frame deletion is analogous to sporadic mutations that were observed in transgenic mice overexpressing Neu, the rat counterpart of human HER2 (155). The deletion of exon 16 alters the confirmation of HER2 at the juxtamembrane region and potentially exposes a free cysteine that facilitates the formation of intermolecular disulfide bond, thereby promoting constitutive homodimerization of the receptors (156). Compared to wild type HER2, this variant has greater transformation capability when ectopically expressed in NIH3T3 cells (118) and higher tumorigenic potential in mice xenograft of HEK293 cells (119) or in transgenic mice where Δ16HER2 is driven by the MMTV promoter (122). Expression of Δ16HER2 in MCF7 cells induced tamoxifen resistance via the downregulation of mir-15/a/16,

which in turn results in the upregulation of BCL-2 to promote survival (121). Expression of Δ16HER2 in MCF10A cells also induced EMT *in vitro* and an higher incidence of lung metastasis *in vivo* (123). In an elaborate *in vitro* and *in vivo* study, Turpin and colleagues have shown that Δ16HER2 activates a unique signaling cascade to promote a tumor microenvironment that enables greater metastasis (124).

Many of these mechanisms of resistance suggest that simply blocking HER2 dimerization and activation is ineffective in the long-term. There are multiple converging mechanisms to promote reactivation of HER2 signaling and to sustain HER2 phosphorylation in the presence of therapeutic antibodies and inhibitors. HER2 remains the preferred signaling receptor in cancer with HER2 amplification, even when HER2 activation is blocked and signaling through alternative receptors is possible. Therefore, the ablation of HER2 protein by RNA interference should prevent resistance mechanisms such as receptor cross talk, extracellular domain shedding, kinase domain mutations, or splice variant such as Δ16HER2.

RNA Interference

RNA interference or RNAi is a mechanism of gene silencing mediated by a short double stranded RNA (dsRNA). RNAi was originally discovered as a genetic phenomenon in plants in the early 1990's (157). The introduction of an exogenous dsRNA for inducing endogenous gene silencing was first demonstrated in C. elegans in 1998 (158). In the few years that followed, the mechanism of RNAi was elucidated with the discovery of the various components of the RNAi machinery.

Today, RNAi is used extensively in molecular biology research, from basic science to clinical applications as a therapeutic.

Cells use RNAi to regulate endogenous gene expression post-transcriptionally or to defend against viral infection by cleaving foreign RNA molecules. In the endogenous microRNA (miRNA) pathway, RNA polymerase II first transcribes primary microRNAs (pri-miRNAs) from DNA (159), which are then processed by the class 2 ribonuclease III Drosha to pre-miRNAs (160). Pre-miRNAs are exported out of the nucleus via the exportin-5 transporter (161, 162). The cytoplasmic ribonuclease III, Dicer, cleaves pre-miRNAs into 22-nucleotide mature miRNAs (163). The mature miRNA is loaded onto the RNA-induced silencing complex (RISC) (164), which unwinds the RNA duplex and selects the strand that is complementary to the target mRNA, the guide or antisense (AS) strand and discards the sense (S) or passenger strand (165, 166). RISC then mediates target mRNA recognition and subsequent translational repression or mRNA cleavage by Argonaute 2 (AGO2) (167, 168). MiRNAs have stem-loop structures and are partially complementary to their target mRNAs. Therefore, target recognition is mediated through binding of nucleotides 2 – 8 from their 5’ end, the seed region, and the 3’ untranslated region (UTR) of their target mRNA (169, 170). As a result, miRNAs typically downregulate the expression of not one, but multiple genes (170, 171). Figure 1.5 shows the processing of miRNA, small interfering RNA (siRNA), and small hairpin RNA (shRNA) for inducing gene silencing

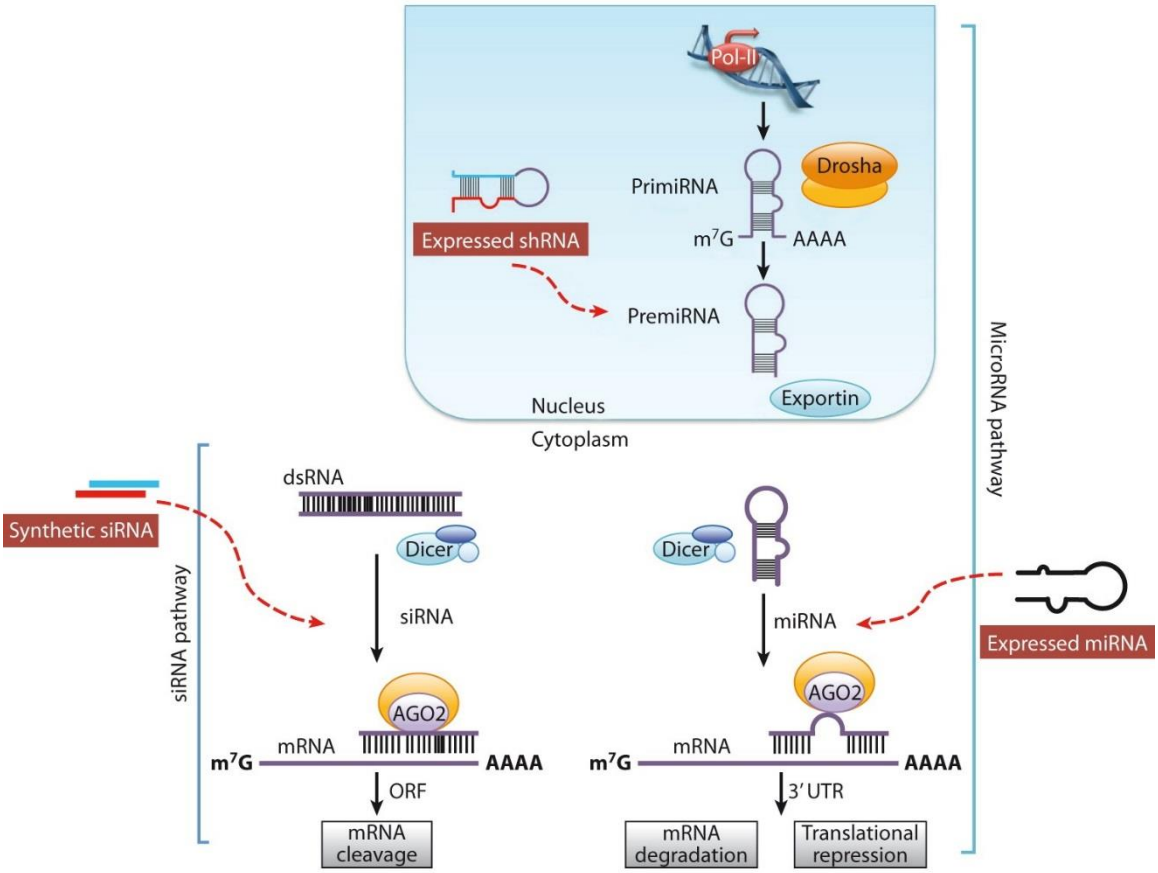

Figure 1.5 RNAi pathways. After processing, miRNA, siRNA, and shRNAs are loaded onto RISC, which discard the sense strand and recognizes the target mRNA. Gene silencing is mediated through translational repression or mRNA degradation. Reprinted with permission from (172).

Exploiting the RNAi machinery

The endogenous RNAi machinery can be harnessed by introducing different types of RNA duplexes to enter and trigger the RNAi pathway along the various stages of processing. Each has their own advantages, depending on the application.

SiRNA and miRNA mimics

siRNAs and miRNA mimics are 19 – 23-nucleotide RNA duplexes that are directly loaded onto RISC upon entry into cells. These RNA molecules are typically introduced into cells via cationic lipid or polymer based transfection reagents or via electroporation. They have transient effects because they are not integrated in the genome of the target cells and therefore cannot be replicated. Unlike miRNA mimics and their endogenous counterparts, siRNAs are designed to be perfectly complementary to the target mRNA sequence.

DsiRNA

Dicer substrate RNAs (DsiRNAs) are 25 – 30 nucleotides long and require Dicer processing prior to RISC loading. While the resulting RNA duplexes are identical to a 21-nucleotide siRNA, DsiRNAs can be more potent than their siRNA counterparts (173). Evidence so far suggests that Dicer not only processes longer dsRNA, but also mediates loading of processed RNA onto RISC and subsequent guide strand selection and orientation (174, 175). However, there are additional design challenges for ensuring proper Dicer processing to yield the correct 21-mer. In addition, the expression of Dicer varies among different types of tumor (176), which adds another layer of complexity for therapeutic use.

ShRNA

ShRNAs are delivered as plasmids or via viral vectors to integrate into the target cell genome. RNA polymerase III transcribes it to a RNA duplex that is

similar to pre-miRNA, but does not require the Drosha processing step (177). Therefore, shRNAs have long lasting effects and can also be designed to be inducible by an external factor, such as an antibiotic or growth factor.

SiRNA design

The canonical synthetic siRNA is a RNA duplex of 19 nucleotides with 2-nucleotide 3' overhangs on each strand, mimicking the product of Dicer processing (178). The 3' overhangs are required for the loading of the siRNA onto RISC because AGO2, the catalytic enzyme that cleaves target mRNA, recognizes these overhangs and ensures the proper orientation of the siRNA (179). While siRNA can be designed to bind virtually anywhere along the mRNA, the silencing efficacy is not the same among all the possible 19-mers that cover the length of the mRNA. The differences can be attributed to the thermodynamic stability of the siRNA duplex (180). Therefore, siRNA design algorithms normally constrain GC content of the duplex to be between 30 – 70% (181). In addition, siRNA sequences with an A or U at positions 10 and 19 as well as a G or C at position 1 also appear to improve the likelihood of getting good silencing efficacy (182).

Off-target effects

Despite the fact that siRNAs are designed to be perfectly complementary to their target mRNA, off-target effects remain common and make it difficult to confirm that the therapeutic effect is the result of silencing the targeted gene, rather than knocking down unwanted genes (183, 184). First, the RNAi machinery has

tolerance for base mismatch and can induce cleavage of the target mRNA without perfect sequence complementarity (184). Second, the passenger strand can sometimes be retained on RISC and used as a template instead of the guide strand, since strand selection is largely determined by the thermostability at the 5' end of the strand, where the less stable one is selected (185, 186). Third, binding of the seed region of siRNA to the 3' UTR of mRNA can trigger a miRNA like repression of multiple genes (187-189). The Basic Local Alignment Search Tool (BLAST) is commonly used to search for potential off-targets following initial design of the siRNA duplex.

In vivo delivery of siRNA

In vivo delivery of siRNAs faces several biological barriers before their therapeutic potential can be unleashed (190). Unprotected siRNAs are quickly degraded in the blood by nucleases and cleared through the kidney. The few that survive degradation and clearance still need to cross the endothelial wall. Upon reaching their target cells, the negatively charged siRNA are not easily up taken by the target cells because the cell membrane is also negatively charged. To overcome these barriers, siRNAs can be loaded onto delivery vehicles such as nanoparticles.

Nanoparticles

Nanoparticles encompass a large family of carriers with diverse chemical compositions. They can be designed and extensively functionalized to meet the

various criteria necessary for the optimal delivery of siRNA. Nanoparticles suitable for drug delivery should have sizes between 20 to 200 nanometers (191). This range not only allows the particles to avoid rapid renal clearance but also cross the fenestrated endothelial barrier to reach their target cells (192). Polyethylene glycol (PEG) is frequently used to coat nanoparticles (193) to avoid clearance by the mononuclear phagocyte system (MPS), also known as the reticular endothelial system (RES) (194), by preventing IgG adsorption and complement activation (195). Nanoparticles reach their target site in a passive manner, relying on the enhanced permeability and retention effect, in which endothelial walls are leakier near tumors than around normal tissue (196, 197). However, this is not always the case as some cancers, such as pancreatic cancer, have an unusually dense stroma that hinders the delivery of therapeutics (198). Targeting molecules, such as ligands or antibodies, can be conjugated to nanoparticles to enhance specific binding to tumor cells and facilitate cellular uptake.

Depending on the size and chemical composition, nanoparticles enter cells primarily through macropinocytosis or endocytosis. Endocytosis can be mediated in a clathrin or caveolin dependent or independent manner (199). Most studies on the uptake and release of nanoparticles have focused on lipid nanoparticles since they have advanced the most clinically. Cationic lipid nanoparticles were found to enter cell via macropinocytosis in a clathrin and caveolin independent manner. They rely heavily on the polymerization of actin filaments as silencing of Cdc42 and Rac1 led to an 80% decrease in uptake while knocking down of clathrin or caveolin effector proteins CHC and Cav1 had minimal impact (200). Ionizable lipid

nanoparticles, on the other hand, appear to require both clathrin mediated endocytosis and macropinocytosis for maximum uptake. When circulating in the bloodstream, they are coated with apolipoprotein E (ApoE) and will subsequently bind to low density lipoprotein receptor (LDLR) to enter cells via receptor-mediated endocytosis (201).

Upon entry into cells, nanoparticles are localized within endosomes, which undergo a maturation stage. The pH of the compartment is gradually lowered, from 6.5 in early endosomes to 4.5 when they fuse with lysosomes (202). Endosomal release of charged nanoparticles with buffering capacity is thought to rely on the proton-sponge effect. Nanoparticles with protonable amines can cause rapid influx of hydrogen ions into the endosome, which in turn induces influx of chloride ions that lead to osmotic imbalance and ultimately results in the bursting of the compartment (203). Endosomal release appears to be a major limiting factor in the efficacy of siRNA. Using fluorescence and electron microscopy, it was estimated that only 3% of up-taken siRNAs are released from endosomes (204) and loaded onto RISC (201). Figure 1.6 provides an overview of targeted delivery of siRNA using nanoparticles.

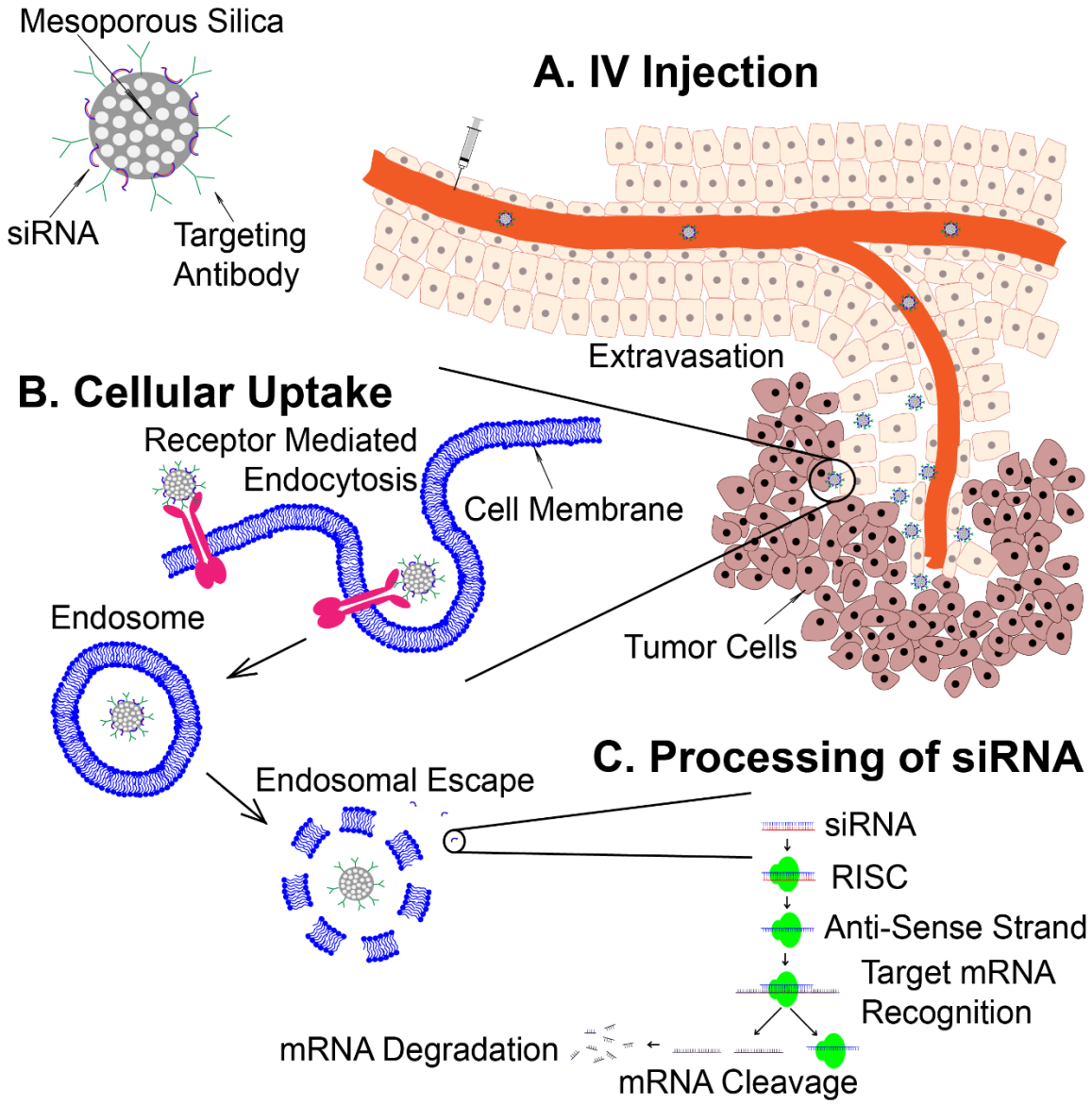

Figure 1.6 Targeted delivery of siRNA by nanoparticles. Intravenously injected siRNAs on nanoparticles travel to the tumor and extravasate through the leaky blood vessels. Targeting moieties such as antibodies allow the particles to enter tumor cells via receptor-mediated endocytosis. During maturation of the endosome, siRNAs are released into the cytosol, which can then enter the RNAi pathway to mediate cleavage of the target mRNA.

Polymeric nanoparticles

Cationic polymers such as polyethylenimine (PEI) and polyamidoamine (PAMAM) readily condense upon contact with anionic nucleic acids through electrostatic interaction to form polyplexes (205). PEI comes in linear and branched forms and has been extensively used for *in vitro* and *in vivo* delivery of siRNA (206). The numerous secondary amines of PEI offer high buffering capacity, which facilitates endosomal release via the proton-sponge effect (203, 207). PAMAM is a well-characterized dendrimer, a type of polymer that forms repeated branches from a central core. One advantage of dendrimers is that their size and shape can be precisely controlled during synthesis. In addition, dendrimers can be easily functionalized by conjugating a diverse range of ligands and peptides, thanks to the presence of various chemical functional groups (208).

Chitosan is derived from deacetylation of the naturally occurring linear polysaccharide chitin, found in the shell of crustaceans and cell wall of fungi. It is highly biocompatible and readily binds siRNA due to its cationic nature. This polymer also contains free amino groups for conjugation of ligands and targeting antibodies (209).

Cyclodextrin is a cyclic polysaccharide with a hydrophobic interior and a hydrophilic surface, which allows for the loading of lipophilic cargo within its cavity (210). The first clinical trial of targeted delivery of siRNA using nanoparticles was based on modified cyclodextrin (211). The experimental therapeutic, CALAA-01, used transferrin as a targeting molecule to deliver RRM2 siRNA to human cancer cells (212, 213).

Polylactic acid-co-glycolic acid (PLGA) has long been used in medical products since the late 1960's. It is highly biodegradable because it can be easily metabolized to lactic and glycolic acid (214). PLGA has high loading capacity and sustained but slow release property (215).

Lipid nanoparticles

Cationic lipids, like polymers, self-assemble when mixed with nucleic acids. They are biocompatible and easily biodegradable, making them a popular choice for delivery of siRNA (216). The use of neutrally charged lipid has also been successful in delivering siRNA to tumors in pre-clinical studies. One formulation that delivered EphA2 siRNA moved on to a phase 1 clinical trial for safety study in patients with solid tumor (217). More advanced formulations employ several different types of lipids to maximize stability and enhance cellular uptake. Examples include stable nucleic acid lipid nanoparticles (SNALP) (218), solid lipid nanoparticles (SLN) (219) and lipidoids (220). However, lipid nanoparticles accumulate primarily in the liver. Delivery platforms based on lipid nanoparticle are now mostly being tested clinically for treating diseases of or associated with the liver (e.g., transthyretin amyloidosis). Clinical trials investigating delivery to solid tumors outside the liver have not been as successful (221).

Inorganic nanoparticles

Inorganic particles include quantum dots and particles with core composed of iron oxide, gold, calcium phosphate, silica, etc. Iron oxide nanoparticles are

superparamagnetic and can be used as contrast agent in MRI (222). They are often coated with cationic polymers for binding of siRNAs. Gold particles are non-toxic and biologically inert, making them an attractive platform for in vivo use (223). Recent studies have investigated the photothermal property of nanoparticles with gold as the outer shell layer. Irradiation with near infrared laser causes the gold layer to resonate and generate heat, which induces cavitation within endosomes and aides with the endosomal escape of siRNA (224, 225). Quantum dots are fluorescent semiconductors that can be used for diagnostics in addition to delivering siRNAs (226). However, toxicity remains a concern because quantum dots commonly contain heavy metals in the form of lead sulfide or cadmium selenide (227). Calcium phosphate is another naturally occurring compound that is highly biocompatible and biodegradable. Once inside the endosome, it is easily dissolved and releases siRNAs as the pH drops. However, calcium phosphate readily aggregates following synthesis and polymer and lipid coatings have been used to maintain its original size to achieve optimal pharmacokinetics and efficacy (228). Silica nanoparticles have several properties ideal for drug delivery. They have low toxicity and a porous structure with large surface area for drug loading (229, 230). Because they are negatively charged, a cationic polymer can be added for binding nucleic acids (231).

RNAi mediated HER2 silencing in cancer

A number of studies have investigated the effect of silencing HER2 in HER2+ cancer cells. Yang and colleagues used a retrovirus to deliver HER2 siRNA

to breast and ovarian cancer cells (232). Their HER2 siRNA inhibited growth and induced apoptosis and cell cycle arrest at the G1 phase. In their xenograft model, HER2 siRNA significantly reduced tumor growth. Faltus and colleagues reported similar findings when they delivered HER2 siRNA *in vitro* using a cationic lipid based commercial transfection reagent (233). Another group demonstrated that the co-silencing of HER2 and PTK6 using shRNA inhibited the growth of JIMT1, a HER2+ cell line that is intrinsically resistant to trastuzumab and lapatinib (234). PTK6 is a non-receptor tyrosine kinase that is often co-expressed with HER2 (235, 236). The simultaneous knock down of HER2 and PTK6 was found to have additive effects *in vitro* but not *in vivo* (234). One group investigated the targeted delivery of a HER2 antisense oligonucleotide (AON) using a trastuzumab conjugated polymalic acid-based polymer (237). The study showed the combined therapeutic effect of trastuzumab and the HER2 AON in a trastuzumab sensitive xenograft model derived from BT474 cells. Unlike RNAi, antisense oligonucleotides are single stranded DNA or RNA molecules that rely on RNase H for mRNA cleavage (238).

These studies suggest that the silencing of HER2 via RNAi has therapeutic impact on HER2 positive breast cancers. However, with the exception of HER2 AON, these studies did not attempt to develop an actual therapeutic that can be delivered *in vivo*. They did not compare the effectiveness of siRNA against that of traditional HER2 targeted therapies, especially in resistant cancers. In addition, no studies have investigated whether cancer cells develop resistance to RNAi the way they do to therapeutic antibodies or small molecule inhibitors.

Mesoporous silica nanoparticles for siRNA delivery

Our group has developed a mesoporous silica nanoparticle (MSNP) delivery system with a core size of approximately 50 nm (239). A layer of cross-linked PEI is added to bind siRNAs and to facilitate endosomal escape via the proton-sponge effect. The construct is then decorated with PEG to reduce clearance by phagocytes, shield siRNA from nucleases, and reduce toxicity associated with PEI. In addition, antibodies are conjugated at the end of PEG chains to specifically target cancer cells, while reducing the potential off-target delivery of siRNAs to normal cells.

We have previously demonstrated the benefits of using our MSNP for siRNA delivery (239). Our study showed that siRNAs on MSNPs had a much longer half-life in serum compared to free, unprotected siRNAs. SiRNAs on MSNPs remained intact for up to 24 hours while free siRNAs were degraded in under 30 minutes. This is remarkable when benchmarked against the siRNA-cyclodextrin material that went to clinical trial having only 60% protection for 5 hours (240). The PEG layer was responsible for protection against serum nucleases as siRNAs on PEI-MSNP without PEG were rapidly degraded.

To show that targeting antibodies facilitate the specific cellular uptake of MSNPs, we used flow cytometry to compare the uptake efficacy of MSNPs with HER2 antibodies and those with CD20 antibodies in HER2+ cancer cells (239). CD20 proteins are expressed only on B cells. After 2 hours of incubation with these two types of MSNPs loaded with fluorescently labeled siRNAs, greater than 90% of the cells have internalized MSNPs with HER2 antibodies, while less than 10%

have internalized those with CD20 antibodies. In addition, cellular uptake was minimal in cells that did not express HER2 receptors.

In the same report, we also investigated the safety profile of our MSNPs with respect to blood compatibility and immune response. *In vitro* assays showed that our MSNPs with and without siRNAs did not induce hemolysis, abnormal blood clotting, or platelet aggregation. After incubating with peripheral blood mononuclear (immune) cells, our MSNPs loaded with siRNAs did not trigger the release of inflammatory cytokines associated with an immune response to foreign antigens such as nucleic acids.

In our biodistribution and pharmacokinetics studies (unpublished), our MSNPs prolonged the circulation of the loaded siRNAs by 11.8 fold over free siRNAs following i.v. injection into immunocompetent mice. We obtained similar results in immunodeficient mice, indicating that our MSNPs triggered little to no immune response that would have otherwise affected the pharmacokinetics. In mouse xenograft models of human breast cancer, MSNPs without targeting antibodies enhanced the accumulation of siRNAs in tumors by 10 fold solely relying on the effect of EPR. Serum biochemistry and histology showed good liver and kidney safety in mice that received multiple doses of MSNPs with targeting antibodies and siRNAs. In contrast to insoluble silica particulates, which may cause lung fibrosis, our nano-sized MSNPs readily dissolve at body pH to silicic acid, which can be cleared by the kidneys through urine (241).

In all, our MSNP is an effective siRNA delivery platform with excellent *in vitro* and *in vivo* safety profiles.

Scope of this dissertation

The goal of this dissertation is to develop a nanoparticle delivered siRNA therapeutic targeting HER2, characterize its anti-proliferative activity *in vitro* and *in vivo*, and demonstrate its superior efficacy over the current HER2 targeted therapies, trastuzumab and lapatinib. I also investigate whether cancer cells develop resistance to siRNA mediated silencing of HER2 when treated over an extended period.

Chapter 2 describes the design of the siRNA and the screening process to determine the most potent sequence among a pool of 76 candidates that covers the entire HER2 coding region. This sequence was selected based on the silencing and anti-proliferative efficacy in a panel of HER2+ cell lines, as well as the number of potential off-targets and effect on HER2- cell lines. I then characterize the selected siRNA sequence *in vitro* with respect to its effect on cell cycle, apoptosis and downstream signaling pathways. I also show that HER2 silencing can overcome intrinsic and acquired resistance in cells that are natively resistant to trastuzumab or lapatinib or cells that were selected to become resistant under prolonged treatment. Using the mesoporous silica nanoparticles developed in our lab, HER2 siRNA was delivered to trastuzumab resistant HER2+ tumors in a mouse xenograft model. By monitoring tumor growth over time, I show that the tumors still respond to HER2 siRNA but not to trastuzumab.

In chapter 3, I investigate the effect of repeated HER2 silencing and show that cancer cells do not develop significant resistance within the same period that they do to trastuzumab or lapatinib. In addition, I demonstrate that repeated HER2

siRNA treatments do not lead to epithelial to mesenchymal transition or an increase in the number of tumor initiating cells in the population. Using reverse phase protein array, I show that HER2 signaling remains suppressed under siRNA while lapatinib resistant cells upregulate HER2 signaling even in the presence of the inhibitor.

Chapter 4 concludes this dissertation by summarizing my research and proposing future directions that can be derived from my findings.

CHAPTER 2

THERAPEUTIC SIRNA FOR DRUG-RESISTANT HER2-POSITIVE BREAST CANCER

This chapter was originally published in Oncotarget. 2016;7(12):14727-41.

Abstract

HER2 is overexpressed in about 20% of breast cancers and contributes to poor prognosis. Unfortunately, a large fraction of patients have primary or acquired resistance to the HER2-targeted therapy trastuzumab, thus a multi-drug combination is utilized in the clinic, putting significant burden on patients. We systematically identified an optimal HER2 siRNA from 76 potential sequences and demonstrated its utility in overcoming intrinsic and acquired resistance to trastuzumab and lapatinib in 18 HER2-positive cancer cell lines. We provided evidence that the drug-resistant cancer maintains dependence on HER2 for survival. Importantly, cell lines did not readily develop resistance following extended treatment with HER2 siRNA. Using our recently developed nanoparticle platform, systemic delivery of HER2 siRNA to trastuzumab-resistant tumors resulted in significant growth inhibition. Moreover, the optimal HER2 siRNA could also silence an exon 16 skipped HER2 splice variant reported to be highly oncogenic and linked to trastuzumab resistance.

Introduction

Breast cancer is one of the leading causes of death among women in the United States. Among various subtypes of breast cancer, overexpression and amplification of the human epidermal growth factor receptor type 2 (HER2; *ErbB2/neu*) accounts for about 20% of all cases and is a predictor of aggressive phenotype and poor prognosis (242). HER2, a member of the HER family, is a transmembrane receptor tyrosine kinase and has been well characterized as an oncogenic driver of human breast and ovarian cancer (243). Activation of HER2 downstream signaling requires either self-dimerization or dimerization with other HER family members (18). Downstream signaling is mainly mediated through activation of AKT and ERK pathways, leading to cellular proliferation and survival (244). Overexpression of HER2 increases downstream activity by increasing the probability of homo- and heterodimer formation (245) and/or by increasing the production of a highly oncogenic HER2 splice variant that lacks exon 16 hereafter referred to as delta16 HER2 (118).

FDA-approved targeted therapies for HER2-positive breast tumors include trastuzumab (Herceptin®, Genentech), pertuzumab (Perjeta®, Genentech), T-DM1 (Kadcyla®, Genentech) and lapatinib (Tykerb®, Norvatis). Trastuzumab and pertuzumab are humanized monoclonal antibodies that bind to the extracellular domain of the HER2 receptor and block receptor dimerization, thus preventing receptor activation (246). T-DM1 is trastuzumab conjugated to the cytotoxic agent emtansine. Lapatinib is a small molecule dual kinase inhibitor that actively blocks HER2 signaling by binding to the HER2 and/or EGFR kinase domain (247).

Despite the use of the aforementioned HER2-targeted therapies, patients with advanced HER2-positive breast cancer still develop resistance to the best combination regimens (e.g., trastuzumab, pertuzumab, and docetaxel) and progression-free survival is still only 18.5 months (248). Clearly, more durable treatments are needed.

Recent studies have shown the potential use of HER2 siRNA as therapeutics for treating HER2-positive breast cancer (233, 249). One group has demonstrated its potential to overcome resistance to trastuzumab (94). However, these works have been limited to *in vitro* studies due to the lack of readily available *in vivo* delivery platforms. In addition, the siRNA sequences used in prior works have not been systematically screened. Some studies have infected cancer cells ex vivo with HER2 siRNA (232) or shRNA (234) prior to tumor inoculation in order to demonstrate the *in vivo* activity of HER2 suppression. However, to make RNAi clinically relevant, effective *in vivo* delivering of siRNA to tumors is necessary. Although viral-based siRNA and shRNA strategies are effective, concerns regarding immunogenic response and insertional mutagenesis remain major issues (250). Recent advances in nanobiotechnology have made non-viral based siRNA delivery viable. Inoue et al. showed that a polymalic acid-based nanobiopolymer conjugated with HER2 antisense and trastuzumab can inhibit tumor growth in BT474 tumor xenografts (237). Two additional studies have utilized siRNA against PLK1 delivered systemically with peptide fusion protein (251) or PLA-PEG (252) to successfully treat BT474 tumors in mice. However, these studies utilized BT474 derived tumors, which are sensitive to trastuzumab.

This article addresses several aforementioned shortcomings in the field. We have identified the most optimal HER2 siRNA duplex from a pool of 76 potential sequences. We then demonstrate the *in vitro* efficacy of the optimal HER2 siRNA to overcome both intrinsic and acquired drug resistance in HER2-positive cancer cell lines, followed by the *in vivo* efficacy by utilizing our recently optimized nanoparticle platform (253) to systemically deliver siRNA to solid tumors. We elucidate that the drug-resistant cancer still relies on HER2 pathways, substantiating the utility of a HER2 siRNA treatment strategy to overcome drug resistance. Importantly, we also address whether HER2-positive cancer can develop resistance to HER2 siRNA after long-term treatment as it does with HER2-targeted therapies. Lastly, we show that our optimal HER2 siRNA can silence both wild-type HER2 and the more oncogenic delta16 HER2 splice variant to the same extent.

Materials and methods

Design and synthesis of HER2 siRNA

Seventy-six siRNA duplexes targeting the coding region of human HER2 (NM_001005862) were designed, each with 3' UU overhangs and no other modifications. Four negative control siRNAs were purchased from commercial sources: 2 non-targeting siRNAs (AllStar Negative Control siRNA from QIAGEN and ON-TARGETplus™ Control Pool from Thermo Scientific Dharmacon®), a Luciferase siRNA and a GFP siRNA. All 76 custom HER2 siRNAs were

synthesized by QIAGEN. A separate non-targeting siRNA, siSCR, was loaded onto the nanoparticle for the *in vivo* study.

Cell culture and transfection of siRNA

The majority of cell lines used in this study were obtained from ATCC and have not been re-authenticated prior to use. JIMT1, EFM192A, EFM192B and EFM192C were obtained from DSMZ. 21PT, 21NT, 21MT1 and 21MT2 were obtained from Dr. Kornelia Polyak (Harvard Medical School). BT474-TRgf was obtained from Drs. Robert Kerbel (University of Toronto) and Giulio Francia (now at University of Texas at El Paso). All media and supplements were purchased from Life Technologies. The growth and medium conditions of breast cancer cell lines have been previously published (254). Cells used in the cytotoxicity were cultured according to ATCC guidelines. Transfection was carried out using DharmaFECT-1 transfection reagent (Thermo Scientific Dharmacon) diluted in OptiMEM medium (Life Technologies). Cells were seeded 24 hours prior to transfection using DharmaFECT-1 at a final dilution of 1:200. DharmaFECT-1 and HER2 siRNA were left in the wells after transfection until assay completion.

For long-term transfection, a final concentration of 1 nM siHER2^{d75} was used throughout the 15-week period. Cells were passaged such that they never exceed 75% confluency. Transfection was repeated weekly. A second set of cells were subjected to negative control siRNA transfection under the exact same conditions. At the end of the 15-week period, both populations were taken off transfection and expanded for experiments.

Establishment of acquired resistant BT474 cell line derivatives

To establish the trastuzumab-resistant BT474-TR, parental BT474 cells were initially maintained in RPMI-1640 complete medium with 10 µg/ml trastuzumab (purchased from OHSU Pharmacy) for 1 month and then the trastuzumab concentration was gradually increased to 30 µg/ml over the next 5 months. To generate the lapatinib-resistant BT474-LR, parental BT474 cells were initially cultured in RPMI-1640 complete medium with 50 nM lapatinib (LC Laboratories, L-4804) for 1 month and then the lapatinib concentration was gradually increased to 1 µM over the next 5 months. The BT474-TR cells were then maintained in 30 µg/ml trastuzumab, while BT474-LR in 1 µM lapatinib for subsequent studies. The resistant cell derivatives were taken off trastuzumab or lapatinib 3 days prior to seeding for experiments.

Quantification of RNA expression

The mRNA levels of HER2 and actin control were measured using QuantiGene® 2.0 Reagent System (Affymatrix Panomics). Briefly, treated and control samples were lysed 5 days after transfection. The lysates were allowed to hybridize overnight with HER2 and actin specific probes on the capture plate. Signal amplification was achieved by further hybridization with Pre-Amplifier, Amplifier and Labeled Probe. The Labeled Probe is conjugated with alkaline phosphatases, which degrade the chemiluminescent substrate added during the last step to produce luminescence. Signals were detected on the GloMax-Multi+ Detection System (Promega). The amount of luminescence is directly proportional

to the amount of mRNA present in each sample. The HER2 mRNA levels of treated and control samples were then normalized to their respective actin mRNA levels. The normalized values from treated samples were divided by the averages values of the 4 control siRNAs to determine the percentage of HER2 knockdown.

RT-PCR was used to detect the mRNA level of wild-type and delta16 splice variants. Primers were previously published (119) but the MGB quencher was used instead of TAMRA on the probes. All primers and probes were ordered from Applied Biosystems (Part of Life Technologies). Total RNA was extracted and purified using the RNeasy Kit (Qiagen). 50 ng of total RNA was used in a one-step RT-PCR reaction ran on the ABI7500 Fast Thermocycler. Cycling conditions were 50 °C for 15 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 sec and 61 °C for 1 min. Data were analyzed using the delta CT method by normalizing to GAPDH.

Cell viability assay, dose response plot and calculation of GI values

Cell viability was determined using the CellTiter-Glo assay (Promega). Cells were seeded in 96-well plates 24 h prior to transfection or drug treatment. Plates were read 3 days after lapatinib treatment or 5 days after trastuzumab treatment or siRNA transfection. Dose response data points were calculated according to the current NCI-60 DTP Human Tumor Cell Line Screen protocol. Percent growth is calculated from this formula:

$$\text{Percent Growth} = \frac{\text{Treated Viability} - \text{Viability at Time 0}}{\text{Vehicle Control Viability} - \text{Viability at Time 0}} \times 100$$

Dose response curves were plotted and GI values were calculated using GraphPad Prism 6 (GraphPad Software).

Cytotoxicity of T-siHER2^{d75}-NP

The cytotoxicity profile of T-siHER2^{d75}-NP was evaluated in four "normal/non-tumorigenic" cell lines, HEK293, HEPG2, MCF10A, and HUVEC were purchased from ATCC and grown in media as recommended by the ATCC. The cells were seeded (cells/well in 96-well plate) at 20,000 (HEK293), 250,000 (HEPG2), 120,000 (MCF10A), and 7,500 (HUVEC), respectively, for 24 h to achieve about 80% confluency. The cells were then treated with efficacious dose of T-siHER2^{d75}-NP (60 nM as siHER2^{d75}, 42 μg/mL as nanoparticle, (253)) or acetaminophen (APAP, Tylenol) as a drug benchmark at 25 mM following the NCL's protocol (255). Cell viability was measured at 24 h post-treatment with the CellTiter-Glo assay, which measures adenosine triphosphate (ATP) as an indicator of metabolically active cells, and reported as percent of the untreated control's viability.

Immunofluorescence imaging

Cells were seeded in 96-well black wall plates (Corning® Costar®) 24 hours prior to transfection. Three days after transfection, cells were fixed in 2% paraformaldehyde for 15 minutes at room temperature and then permeabilized with 0.3% Triton-X. After blocking for one hour in 2% BSA, cells were stained overnight at 4°C with primary antibodies. Secondary antibody incubation was carried out at room temperature for one hour the next day. Nuclei were stained with Hoechst 33342 dye (Life Technologies). Plates were imaged on an Olympus

IX81 Scan^R automated fluorescence microscope. Images were analyzed, and signal intensities were quantitated using the included analysis software package.

Western blot

Cells were seeded in 6-well plates 24 hours prior to transfection or drug treatment. Samples were harvested 48 hours after drug treatment and 72 hours after transfection. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails. Running samples were prepared with 4X Novex NuPAGE LDS sample buffer (Life Technologies) and supplemented with 10% beta-mercaptoethanol. Twenty micrograms of protein were loaded per lane of 4-12% Bis-Tris NuPAGE gels (Life Technologies) and ran in MOPS buffer. Proteins were then transferred to PVDF-FL membrane (Millipore) in Tris-Glycine transfer buffer. Primary antibody incubation was carried out overnight at 4°C on a rocking platform. Secondary antibody incubation was carried out at room temperature the next day. Proteins were detected using the LI-COR Odyssey imaging system (LI-COR Biosciences). Image analysis and protein quantitation were performed with ImageJ (NCI).

Cell cycle analysis

Cells were seeded in 10-cm dishes at 1-2 x10^6 cells per dish. After an overnight settling period, cells were transfected with selected siRNAs. After 48 or 72 hours, cells were harvested and fixed in 70% ethanol. Cells were incubated with 50 µg/ml RNase A in PBS for 30 minutes at 37°C and then stained with 50 µg/ml

propidium iodide. The cells were subsequently analyzed on a BD Calibur flow cytometer. Analysis of cell cycle results was carried out using FlowJo (Tree Star, Inc.).

Apoptosis analysis

Apoptotic cells were detected using Yo-Pro-1 staining. Briefly, BT474 cells transfected with 10 nM siHER2 or siControl were stained with 1 μmol/L of Yo-Pro-1 dye and 10 μg/mL of Hoechst 33342 for 30 minutes at 37°C. The fluorescence dyes were detected by high content imaging (Olympus IX81 Scan^R automated fluorescence microscope). The percentage of apoptotic cells were calculated by the ratio of Yo-Pro-1 and Hoechst positive cells.

Targeted delivery of siHER2^{d75} in a mouse model of HER2-positive breast cancer xenograft

All animals were used under an approved protocol of the Institutional Animal Care and Use Committee (IACUC) of Oregon Health and Science University (OHSU) and the experiments were carried out under the auspices of the Department of Comparative Medicine of OHSU. BT474-TRgf cells (1 x 10^7) were injected subcutaneously into the flanks of six-week-old athymic nu/nu mice (Taconic) and allowed to grow to an average size of ~100 mm^3. Mice were then grouped and proceeded to receive twice a week of siHER2^{d75} or siSCR loaded on our mesoporous silica based nanoconstructs containing trastuzumab (T-NP) for targeted delivery over a period of six weeks via tail vein injection. Days of injection

and doses were specified in Fig. 8B. Details on the nanoconstruct design, composition, and synthesis procedures can be found in our previous report (253). Trastuzumab was administered intravenously twice weekly at a dose of 2.5 mg/kg.

Statistical analysis

All *in vitro* experiments were performed in three or more replicates with results reported as mean ± standard deviation (SD). Data for the *in vivo* experiment were plotted as mean ± standard error of the mean (SEM). Student t test (normal distribution) or Mann-Whitney test (nonparametric, unpaired) was used for group comparison. Multiple comparisons of three or more groups were done using one-way ANOVA (normal distribution) or the Kruskal-Wallis nonparametric test with post-hoc Dunnett multiple comparison tests. GraphPad Prism 6.0 software (GraphPad Software Inc.) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

Results

Screening and validation of HER2 siRNA

Seventy six potential HER2 siRNA candidates plus 2 scrambled siRNAs, an siRNA against luciferase and an siRNA against GFP, were tested for their HER2 mRNA knockdown efficiency in the HER2-positive cell lines BT474, SKBR3 and HCC1954. The 76 siRNAs against HER2 were designed to target sequences distributed across the whole coding region of the human *ERBB2* gene (NM_001005862). All siRNAs had 3’ UU overhangs and no additional

modifications. The sequences of all HER2 siRNAs and control siRNAs are listed in Tables 2.1 and 2.2. The HER2 mRNA levels in HER2-positive breast cancer cell lines (BT474, SKBR3, and HCC1954) were measured following 5 days of transfection with 10 nM of each individual HER2 siRNA sequence (Figure 2.1A).

Table 2.1 Sequences and molecular weights of HER2 siRNAs

siRNA	Target Sequence	Molecular Weight
d1	GCCAGGUGGUGCAGGGAAA	13355
d2	GCUCAUCGCUCACAACCAA	13325
d3	GCACCCAGCUCUUUGAGGA	13340
d4	AUGGAGACCCGCUGAACAA	13325
d5	GGAGACCCGCUGAACAAUA	13325
d6	CUUCGAAGCCUCACAGAGA	13325
d7	CCUCACAGAGAUCUUGAAA	13215
d8	UCACAGAGAUCUUGAAAGG	13333
d9	GGGUCUUGAUCCAGCGGAA	13421
d10	CCCAGCUCUUUGAGGACAA	13293
d11	GCUCUUUGAGGACAACUAU	13294
d12	UCACACUGAUAGACACCAA	13295
d13	GGGAGAGAGUUCUGAGGAU	13325
d14	GGAGAGAGUUCUGAGGAUU	13310
d15	GGAAGGACAUCUUCCACAA	13310
d16	GCUGGCUCUCACACUGAUA	13325
d17	CGUUUGAGUCCAUGCCCAA	13325
d18	GCAUGGAGCACUUGCGAGA	13340
d19	GUGCCAAUAUCCAGGAGUU	13310
d20	CAACCAAGAGGUGACAGCAGAGGAU	17183
d21	GCUCCAAGUGUUUGAGACU	13311
d22	UGUUUGAGACUCUGGAAGA	13295
d23	UGGAAGAGAUCACAGGUUA	13295
d24	GGAAGAGAUCACAGGUUAC	13310
d25	GCAGUUACCAGUGCCAAUA	13304
d26	UUCCAGAACCUGCAAGUAA	13294
d27	CCUGGCAUUUCUGCCGGAGAGCUUU	17198
d28	GGGACCAGCUCUUUCGGAA	13340
d29	GGCCAGAGGACGAGUGUGU	13355
d30	AGGACGAGUGUGUGGGCGA	13355
d31	AGGAGUGCGUGGAGGAAUG	13340
d32	GGGACCAGCUCUUUCGGAA	13340
d33	GAGUAUGUGAAUGCCAGGCACUGUU	17168
d34	GUGUGGACCUGGAUGACAA	13325

d35	CAGUGUGUGGCCUGUGCCCACUAUA	17198
d36	CAGCAGAAGAUCCGGAAGU	13325
d37	CAGAAGAUCCGGAAGUACA	13310
d38	CGGAAGUACACGAUGCGGA	13340
d39	GCAGAUGCGGAUCCUGAAA	13325
d40	AGAUGCGGAUCCUGAAAGA	13310
d41	AUGCGGAUCCUGAAAGAGA	13310
d42	AAGAGACGGAGCUGAGGAA	13325
d43	GGUGAAGGUGCUUGGAUCU	13325
d44	CGGCAGCAGAAGAUCCGGAAGUACA	17198
d45	CCCAAAGCCAACAAAGAAA	13295
d46	CCAAAGCCAACAAAGAAAU	13280
d47	GCAGAUGCGGAUCCUGAAA	13325
d48	CGGAGCUGAGGAAGGUGAAGGUGCU	17213
d49	CGGUGCAGCUGGUGACACA	13355
d50	UGACACAGCUUAUGCCCUA	13310
d51	CUGAACUGGUGUAUGCAGA	13310
d52	GCAGCUGGUGACACAGCUU	13340
d53	AGACAGAGUACCAUGCAGA	13616
d54	GGUCAAGAGUCCCAACCAU	13325
d55	GAUGAUUGACUCUGAAUGU	13280
d56	CGGCCAAGAUUCCGGGAGU	13355
d57	GGGAGUUGGUGUCUGAAUU	13310
d58	GCUUUGUGGUCAUCCAGAA	13303
d59	GCUCACUGCUGGAGGACGA	13355
d60	UGGUGGAUGCUGAGGAGUA	13325
d61	GGUGGAUGCUGAGGAGUAU	13325
d62	CAAAUGUUGGAUGAUUGACUCUGAA	17122
d63	CAGCGCUUUGUGGUCAUCCAGAAUG	17179
d64	GCCAGUCCCUUGGACAGCACCUUCU	17212
d65	CCGAUGUAUUUGAUGGUGA	13295
d66	CCUCUGAGACUGAUGGCUA	13325
d67	GACACUAGGGCUGGAGCCCUCUGAA	17213
d68	GCUGGUGCCACUCUGGAAA	13340
d69	CGGCAGAGAACCCAGAGUA	13340
d70	UGGAAGAGAUCACAGGUUA	13295
d71	GAGACCCGCUGAACAAUAC	13326
d72	GGAGGAAUGCCGAGUACUG	13340
d73	GCUCAUCGCUCACAACCAA	13324
d74	AACAAAGAAAUCUUAGACGAA	14536
d75	CACGUUUGAGUCCAUGCCCAA	14611
d76	GGUGCUUGGAUCUGGCGCUUU	14626

Table 2.2 Sequences of control siRNAs

siRNA	Target Sequence	Manufacturer
Luciferase	CAAGCUGACCCUGAAGUUCUU	QIAGEN
GFP	CGUACGCGGAAUACUUCGAUU	QIAGEN
AllStar	GGGUAUCGACGAUUACAAA	QIAGEN
ON-TARGETplus	Proprietary	Thermo Scientific Dharmacon®
siSCR	UGGUUUACAUGUCGACUAA	Thermo Scientific Dharmacon®

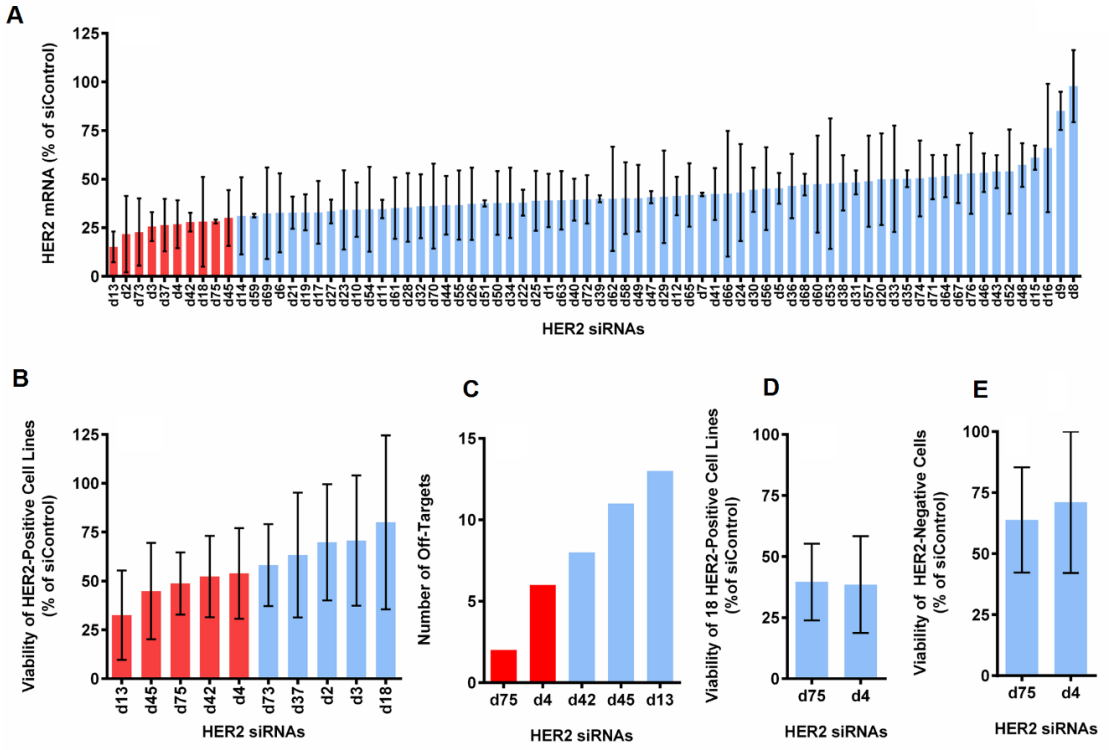

Figure 2.1 Selection of optimal HER2 siRNA sequences based on the level of HER2 mRNA knockdown, cell viability reduction, number of off-targets and specificity. (A) The average expression of HER2 mRNA in 3 HER2-positive cell lines (BT474, SKBR3 and HCC1954), measured by QuantiGene assay at 5 days post-transfection (normalized to actin and reported as the percentage of the average of 4 control siRNAs). (B) The cell viability reduction induced by the top 10

candidates from (A), measured with CellTiter-Glo assay at 5 days post-transfection. Graph shows the average value from 5 HER2-positive cell lines (BT474, SKBR3, HCC1954, HCC1569, and JIMT1). (C) The number of off-targets found through BLAST for the top 5 sequences from (B). (D) Cell viability reduction induced by the best 2 sequences from (C) in 18 HER2-positive cell lines (Figure 2.2). (E) Cell viability reduction induced by the best 2 sequences in 2 HER2-negative cell lines (T47D and MCF10A). All with 10 nM of siRNA delivered with DharmaFECT-1.

We retested the ability of the ten sequences with the highest knockdown efficiency to inhibit growth of BT474, SKBR3, HCC1954, HCC1569, and JIMT1 (Figure 2.1B). The five most effective sequences from this evaluation were further ranked according to the number of off-target matches by utilizing BLAST (BLASTN 2.2.31+, RefSeq, NCBI Reference Sequence Database) with a minimum threshold of 70% sequence homology (Figure 2.1C). The sequences d75 and d4 had the fewest off-target matches and were further validated in a larger panel of 18 HER2-positive cell lines (Figure 2.1D and Figure 2.2A) and 2 HER2-negative cell lines (Figure 2.1E). Both sequences yielded comparable cell killing in the 18 cell lines and showed similar treatment specificity (i.e., were less likely to kill HER2-negative cells). However, d75 had fewer off-target matches than d4 (2 vs. 6) and d75 generated more consistent mRNA knockdown and cell viability reduction among the HER2-positive cell lines tested. Low off-target was also apparent when tested in T47D (HER2-negative) cell line, in which GI50 was not achieved even when the concentration of d75 was increased to 80 nM (Figure 2.2B). We selected sequence

d75 for further development based on these results. Henceforth, d75 will be referred to as siHER2^{d75} and the AllStar non-targeting control siRNA will be referred to as siControl (Table 2.2).

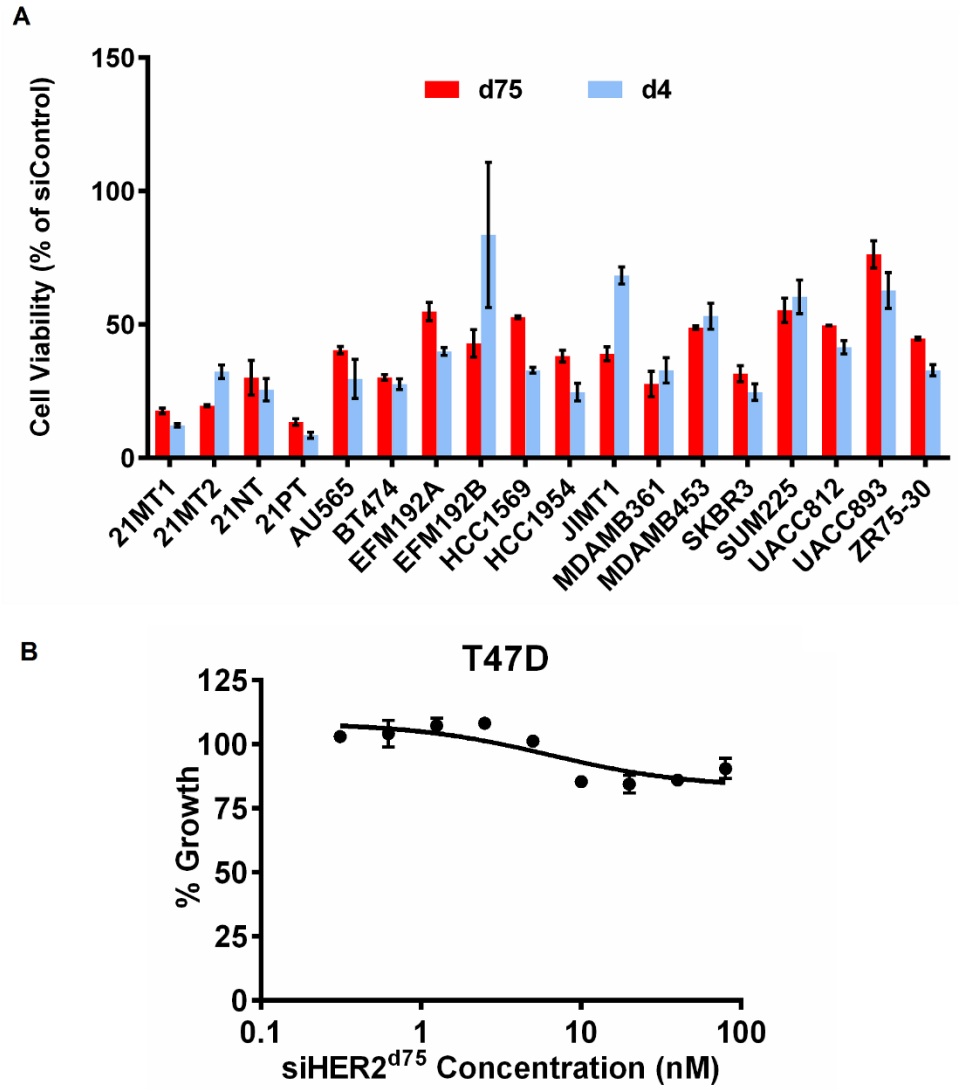

Figure 2.2 Effect of HER2 siRNAs on cell viability in additional HER2+ and HER2- cell lines. (A) The cell viability reduction induced by the top 2 HER2 siRNA candidates from Figure 2.1A – C, measured in HER2-positive cell lines using CellTiter-Glo assay at 5 days post-transfection. All with 10 nM of siRNA delivered

with DharmaFECT-1. (B) Low off-target effect of siHER2^{d75} was confirmed in T47D (HER2-negative) cell line, in which the GI50 was not achieved even when the siHER2^{d75} concentration was increased to 80 nM.

The ability of siHER2^{d75} to decrease HER2 protein levels and induce apoptosis and cell cycle arrest was assessed subsequently (Figure 2.3). Visual and quantitative analyses of the intensity of anti-HER2 immunofluorescence staining before and after treatment with siHER2^{d75} showed that HER2 protein levels on the cellular membrane and in the cytoplasm decreased significantly in BT474 cells after 10 nM siHER2^{d75} treatment for 72 h (Figure 2.3A & B). We confirmed the reduction of HER2 protein levels by western blot analysis (Figure 2.3C). Similar results were also found in SKBR3 and HCC1954 cells (Figure 2.4). The siHER2^{d75} treatment significantly increased the fraction of BT474 cells in the G1 phase of the cell cycle 48 and 72 hours post-treatment compared to siControl ($p<0.001$ and $p<0.014$ for 48 and 72 hours, respectively; Figure 2.3D – E) and increased apoptotic response by approximately 4-fold over the siControl at 72 h post-transfection ($p<0.018$; Figure 2.3F).

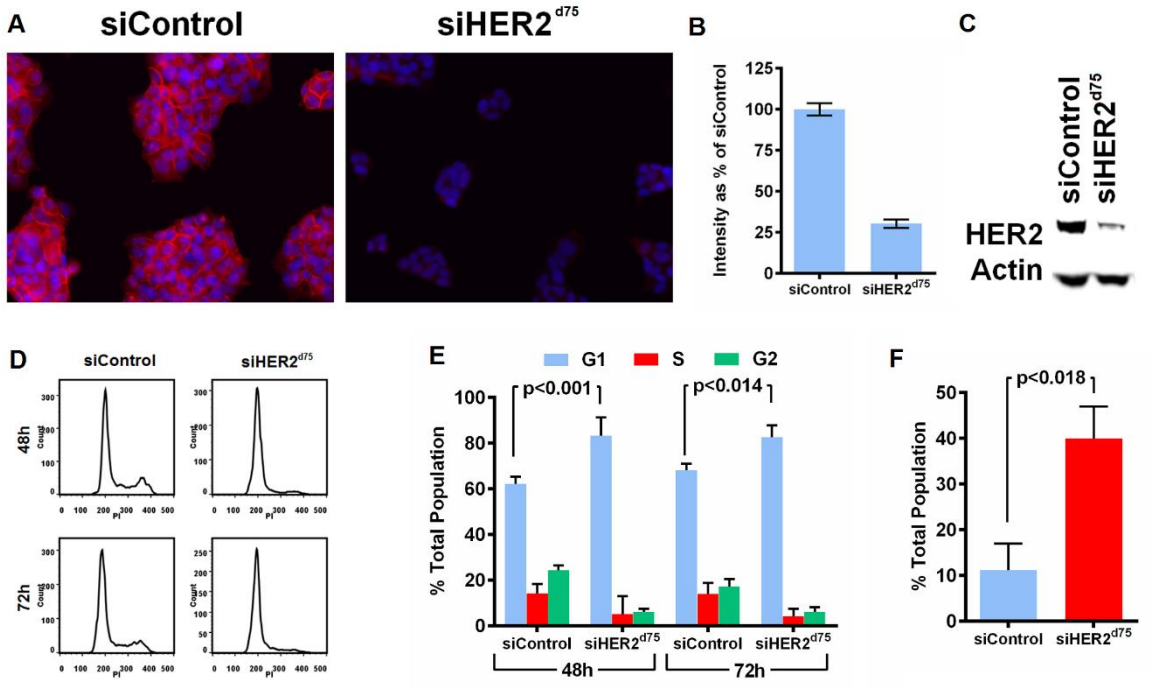

Figure 2.3 Effect of siHER2^{d75} on HER2 protein expression, cell cycle, and apoptosis in the BT474 cell line. (A) HER2 protein reduction by immunofluorescent imaging (200x magification). (B) Corresponding quantified mean signal intensity of HER2. (C) HER2 protein reduction analyzed by western blot. Cell were transfected with 10 nM siHER2^{d75} or siControl for 72 h prior to analysis. (D) Flow cytometry analysis of the treated cells stained with propidium iodide (PI) after 48 h or 72 h. (E) The corresponding percent distribution of cell cycle analyzed using FlowJo. (F) The percent apoptotic cells measured by YO-PRO-1 staining of treated cells after 72 h.

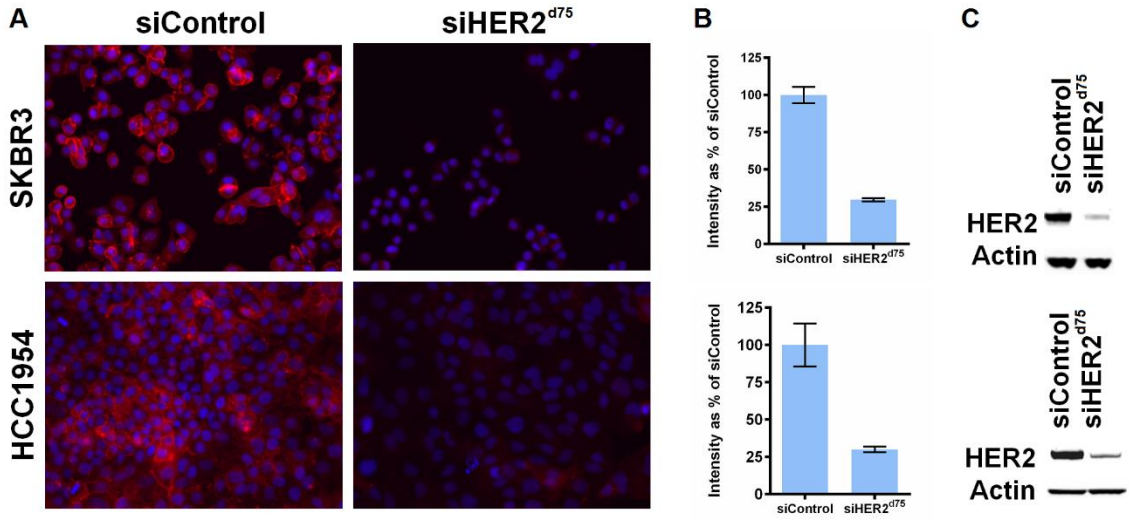

Figure 2.4 Effect of siHER2^{d75} on HER2 protein level in SKBR3 and HCC1954 cells. (A) HER2 proten reduction by immunofluorescent imaging (200x magification), (B) corresponding quantified mean signal intensity of HER2, and (C) HER2 protein reduction analyzed by western blot in SKBR3 and HCC1954 cells. Cells were transfected with 10 nM siHER2^{d75} or siControl for 72 h prior to analysis.

Overcoming intrinsic resistance to trastuzumab and lapatinib with siHER2^{d75}

BT474, HCC1954 and JIMT1 breast cancer cell lines were used to benchmark responses to HER2-targeted therapies because they exhibit different sensitivities to trastuzumab and lapatinib *in vitro*. They also represent HER2 amplification in both the luminal and basal subtypes. The concentrations of trastuzumab and lapatinib needed to inhibit growth by 50% (GI50) in Figure 2.5A & B are summarized in Table 2.3. BT474 was sensitive to both trastuzumab and lapatinib with GI50 values of 0.12 µg/ml and 0.05 µM, respectively. However, JIMT1 was highly resistant to both HER2-targeted therapies, and HCC1954 showed a marginal response to lapatinib (GI50 of 0.39 µM) and was largely

resistant to trastuzumab. Although lapatinib showed some ability to inhibit growth in all three cell lines, the dose required to inhibit the resistant cell lines (HCC1954 and JIMT1) exceeded 2 μM, which starts to be toxic to some HER2-negative cells (58). Not surprisingly, differential sensitivity to siHER2^{d75} was also observed across the three cell lines (Figure 2.5C, with representative HER2 reduction in Figure 2.5D) as evident in the calculated GI50 values, which ranged from 0.19 to 6.78 nM (Table 2.3). However, an siHER2^{d75} dose of only 10 nM could inhibit the growth of BT474, HCC1954, and JIMT1 by 95%, 80%, and 70%, respectively. This suggests that the intrinsically resistant HCC1954 and JIMT1 cell lines still require HER2 for growth, and silencing of HER2 using siRNA in the low nanomolar range is a viable therapeutic strategy.

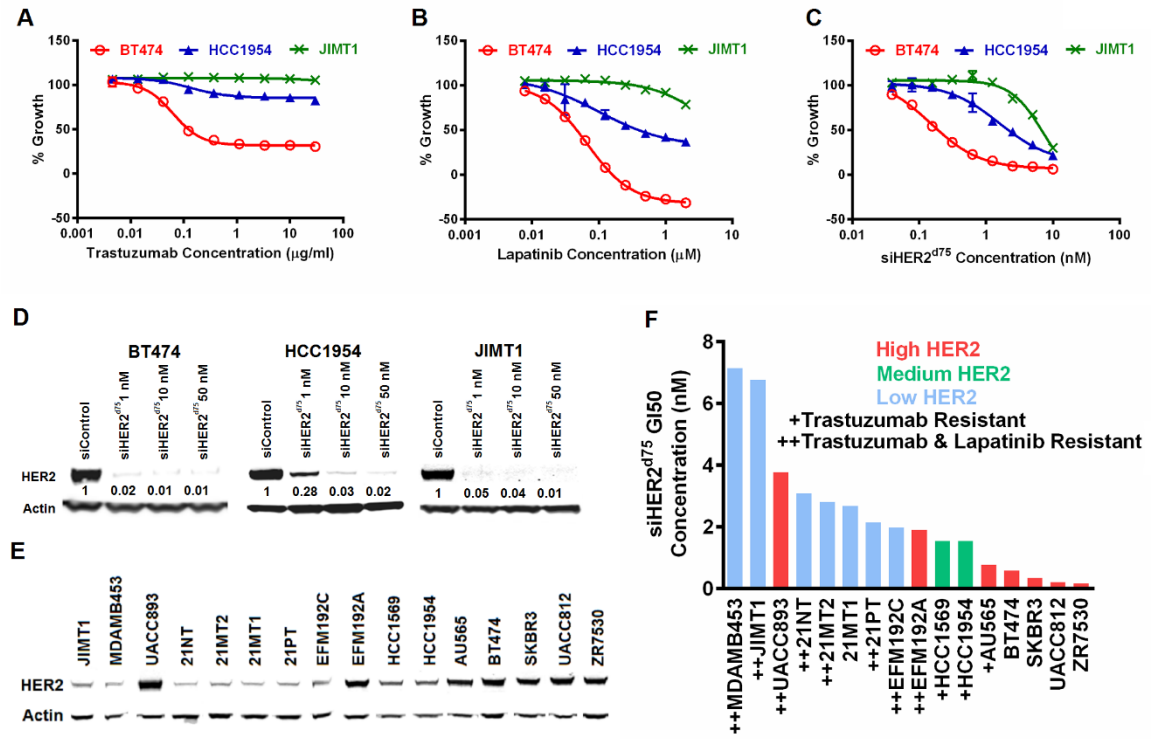

Figure 2.5 Evaluation of growth inhibition with HER2-targeted therapies in representative breast cancer cells. The percent growth of BT474, HCC1954, and JIMT1 breast cancer cell lines treated with (A) trastuzumab, (B) lapatinib, or (C) siHER2^{d75} at the indicated dose range. (D) Reduction of HER2 protein by various doses of siHER2^{d75} vs. 10 nM of siControl after 72 h. Indicated values in (D) represent the fraction of HER2 remaining relative to siControl. (E) Western blot comparing the expression levels of HER2 in 16 HER2-amplified cell lines. Samples were run on 3 separate gels and scanned blots were later combined. (F) Concentration of siHER2^{d75} required to inhibit growth by 50% (GI50) in the 16 cell lines at 5 days post-treatment. “Trastuzumab-resistant” defined as inability to achieve at least 20% growth inhibition at 30 μg/ml dose; “Lapatinib-resistant” defined as inability to achieve at least 20% growth inhibition at 2 μM.

Table 2.3 GI50 concentrations of trastuzumab, lapatinib and siHER2^{d75}

Cell Lines	Trastuzumab (μg/ml)	Lapatinib (μM)	siHER2^{d75} (nM)
JIMT1	>30	>2	6.78 (5.97, 7.60)
HCC1954	>30	0.39 (0.27, 0.58)	2.15 (1.81, 2.60)
BT474	0.12 (0.11, 0.14)	0.05 (0.045, 0.049)	0.19 (0.18, 0.21)
BT474-TR	>30	0.13 (0.12, 0.14)	0.27 (0.20, 0.34)
BT474-LR	>30	1.01 (0.89, 1.15)	0.75 (0.66, 0.87)

Lower and upper limits of 95% CI are given in parentheses.

Previous studies have reported a positive correlation between the efficacy of trastuzumab and lapatinib and the elevated protein expression of HER2 (58, 256). We found that response to siHER2^{d75} in BT474, HCC1954 and JIMT1 was also proportional to HER2 protein expression levels (Figure 2.5E). We investigated the correlation between HER2 protein levels and siRNA induced growth inhibition by measuring GI50 values for siHER2^{d75} in 16 HER2-positive cell lines with varied HER2 levels (Figure 2.5E), many of which were resistant to trastuzumab or both trastuzumab and lapatinib. Indeed, the HER2 protein levels of the 16 cells were largely predictive of their response to siHER2^{d75} as shown in Figure 2.5F. One exception was the UACC893 cell line, which has the highest HER2 expression but only moderately responded to siHER2^{d75} treatment. One possible explanation is that this cell line harbors a PIK3CA^{H1074R} mutation, which can result in AKT activation independent of HER2 (257). Nonetheless, the GI50 values for all these cell lines ranged between 0.15 and 7 nM, thus still supporting the potency of siHER2^{d75} in achieving growth inhibition. In general, HER2 protein level was more predictive of the response to siHER2^{d75} than HER2 mRNA level generated with RNAseq (258) (Figure 2.6).

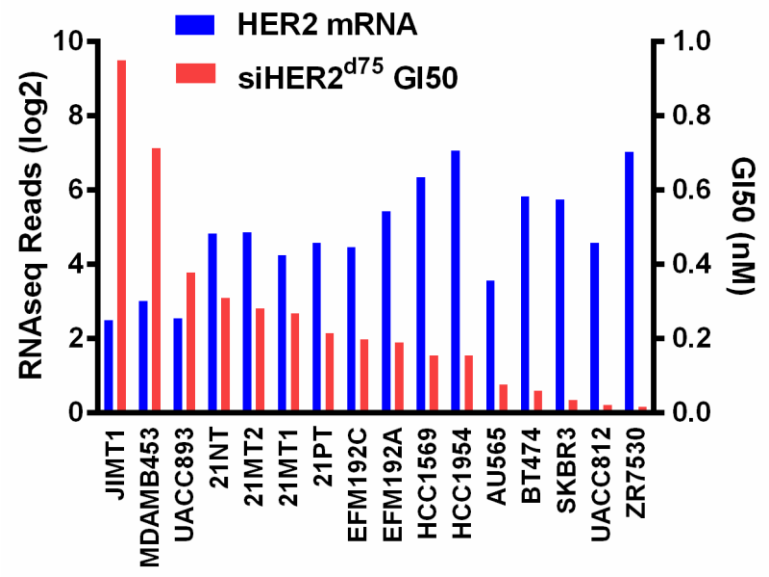

Figure 2.6 Respective GI50 values of siHER2d75 versus HER2 mRNA expression level determined by RNAseq in the 16 HER2-positive cell lines used in the study. Experimental conditions for GI50 measurement are same with Figure 2.5.

Overcoming acquired resistance to trastuzumab and lapatinib with siHER2^{d75}

We used the BT474 cell line to study acquired resistance to trastuzumab and lapatinib. Briefly, BT474 cells were selected in our lab in the presence of increasing concentrations of lapatinib (0.05 – 1 µM) or trastuzumab (10 – 30 µg/ml) over a period of 6 months, similar to the method published by Wang et al. (94). These two cell line derivatives were referred to as BT474-TR for **T**rastuzumab-**R**esistant and BT474-LR for **L**apatinib-**R**esistant. We measured the responses of BT474-TR and BT474-LR to trastuzumab, lapatinib or siHER2^{d75}. BT474-TR was

resistant to trastuzumab (Figure 2.7A) but remained sensitive to lapatinib (Figure 2.7B), while BT474-LR was resistant to both trastuzumab (Figure 2.7D) and lapatinib (Figure 2.7E). Importantly, we did not observe significant resistance to siHER2^{d75} in these two derivatives (Figure 2.7C & F) although the siHER2^{d75} GI50 did increase from 0.19 nM to 0.75 nM in the BT474-LR cell line (Table 2.3).

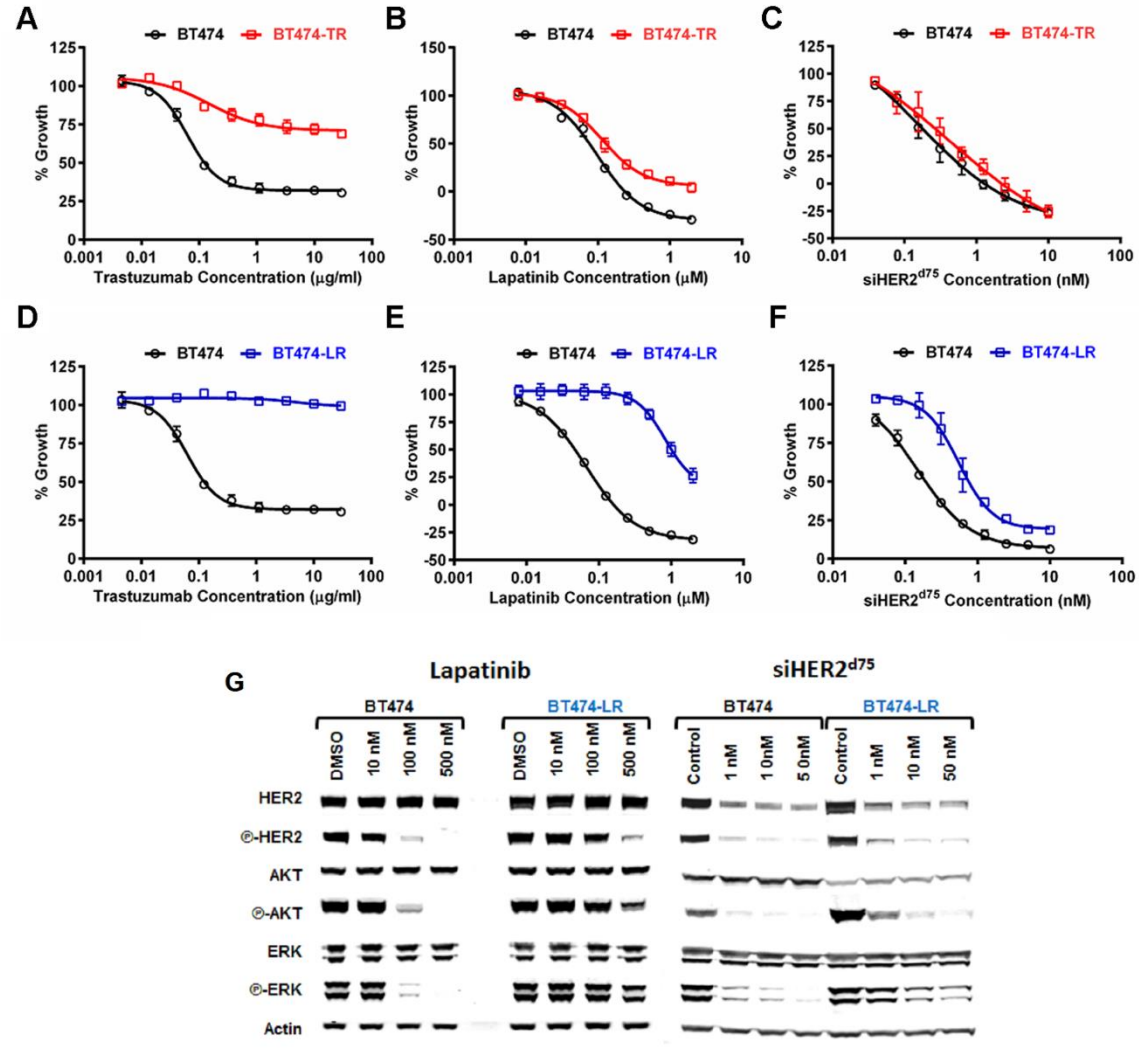

Figure 2.7 Dose response characterization of parental BT474 and BT474 with acquired resistance to trastuzumab (BT474-TR) or lapatinib (BT474-LR) to HER2-targeted agents. (A – C) Growth inhibitory responses of BT474-TR to

trastuzumab, lapatinib, and siHER2^{d75}, respectively. (D – F) Those on BT474-LR. Cell growth was measured at 3 days post-treatment with lapatinib, or 5 days post-treatment with trastuzumab or siHER2^{d75}. (G) Effects of lapatinib and siHER2^{d75} treatment on HER2 signaling pathway of BT474 and BT474-LR cells. Cells were treated with specified doses of lapatinib or siHER2^{d75} for 3 days and the key signaling molecules were analyzed by western blot.

Impact of siHER2^{d75} and lapatinib on phosphorylation status of HER2, AKT an ERK

Lapatinib inhibits HER2 signaling by binding to the HER2 kinase domain and in so doing, decreases phosphorylation of HER2, AKT and ERK (58). We assessed phosphorylation status of HER2, AKT and ERK in the lapatinib-resistant BT474-LR cell line in order to gain mechanistic insight into the cellular responses to increasing doses of lapatinib and siHER2^{d75} treatment (Figure 2.7G). We focused on BT474-LR since it was resistant to both trastuzumab and lapatinib. Untreated BT474-LR displayed a level of phosphorylated HER2 similar to that in the parental BT474. This suggests that BT474-LR cells are still dependent on HER2 signaling. Lapatinib given at 100 nM could block the phosphorylation of HER2, AKT and ERK in the parental BT474 at 72 h post-treatment, whereas a dose of 500 nM was barely able to do so in BT474-LR. On the other hand, 10 nM siHER2^{d75} was effective in BT474-LR displaying reduced phosphorylation of HER2, AKT and ERK to levels that were similar to those achieved in the parental BT474.

This correlates well with our finding (Figure 2.7F) that the sensitivity of BT474-LR to siHER2^{d75} was still on par with that of the parental BT474.

Effect of extended HER2 silencing on BT474 response to trastuzumab, lapatinib, or siHER2^{d75}

We next asked whether HER2-positive cells could develop resistance to siHER2^{d75} after extended treatment. We assessed that by treating BT474, HCC1954 and JIMT1 cells with 1 nM siHER2^{d75} or siControl weekly for 15 weeks. The resulting cell line derivatives were designated as BT474-H15, HCC1954-H15 and JIMT1-H15 (si**H**ER2 for **15** weeks), and BT474-C15, HCC1954-C15 and JIMT1-C15 (si**C**ontrol for **15** weeks). One nanomolar of siHER2^{d75} was sufficient to reduce the expression of HER2 by approximately 98% in BT474, 72% in HCC1954, and 95% in JIMT1 cells (Figure 2.5D). When leaving 1 nM siHER2^{d75} in the medium following transfection, the silencing effect as gauged by HER2 mRNA level reduction lasted for at least 7 days (Suppl. Figure 2.8A). Thus, the siHER2^{d75} transfection was repeated at 7-day intervals when medium change was also needed. Immunofluorescence imaging (Figure 2.8B) confirmed that HER2 reduction occurred uniformly across the BT474 cell population. Because 1 nM siHER2^{d75} did not eradicate the entire cell population, cells that survived under low HER2 conditions were selected over the 15 weeks. After 15 weeks, the remaining cells designated BT474-H15, HCC1954-H15 and JIMT1-H15 were expanded upon the withdrawal of siHER2^{d75} for further characterization. Without siHER2^{d75}, the

rebound of HER2 expression to the level of the naïve cells (never been treated with siHER2^{d75}) was observed within a week (Suppl. Figure 2.8C).

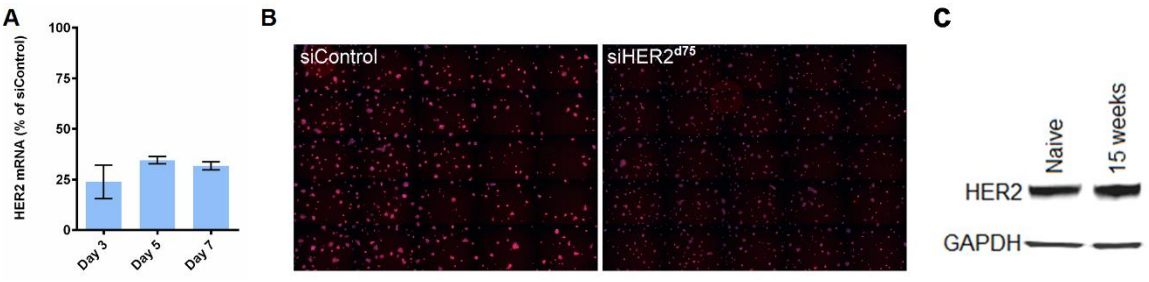

Figure 2.8 HER2 mRNA and protein level after long-term treatment. (A) HER2 mRNA levels in BT474 at days 3, 5 and 7 post-transfection of 1 nM siHER2^{d75}. Indicated values represent the fraction of HER2 remaining relative to siControl. (B) HER2 levels by immunofluorescent detection in BT474 cells treated with 1 nM of siControl or siHER2^{d75} for 72 h. Image is a composite of 25 fields at 100x (each). Red = HER2, Blue = DAPI stained for nuclei. (C) HER2 protein levels in the naïve BT474 cells (i.e., never been treated with siHER2^{d75}) and those treated continuously with 1 nM siHER2^{d75} for 15 weeks (siHER2^{d75} treatment was withdrawn for 1 week prior to western blot analysis).

The responses of the repeatedly transfected cells to varied doses of siHER2^{d75} were not significantly different between the C15 and H15 populations generated from BT474, HCC1954, and JIMT1 (Figure 2.9A – C, respectively). Also, the cells had not developed resistance to either trastuzumab (Figure 2.9D – F) or lapatinib (Figure 2.9G – I) following prolonged siHER2^{d75} treatment, illustrating that cells receiving extended siHER2 treatment still depend on HER2. In contrast, a

slight increase in sensitivity to lapatinib especially at the higher dose range was observed with the H15 populations (vs. C15 counterparts) generated from BT474 and HCC1954 (Figure 2.9G & H).

Figure 2.9J shows the phosphorylation state of signaling molecules in the HER2 pathway of the C15 and H15 populations. The protein profiles of H15 lines before treatment were comparable to those of C15. The apparent slight upregulation of phosphorylation of ERK in BT474-H15 was not significant once normalized to the GAPDH band intensity. Both C15 and H15 cells responded similarly to treatment with siHER2^{d75} in terms of HER2 silencing and inhibition of phosphorylation of HER2, AKT and ERK. Figure 2.9K shows that lapatinib and trastuzumab were able to inhibit phosphorylation of AKT and ERK in BT474-H15. The effect of trastuzumab was less pronounced than that of lapatinib, which has previously been shown to be more effective at blocking HER2 signaling (94). Collectively, these results suggest that HER2-positive cancer cells still rely on the HER2 signaling pathway after extended siHER2^{d75} treatment and are much less prone to develop resistance to siHER2^{d75} than to trastuzumab or lapatinib.

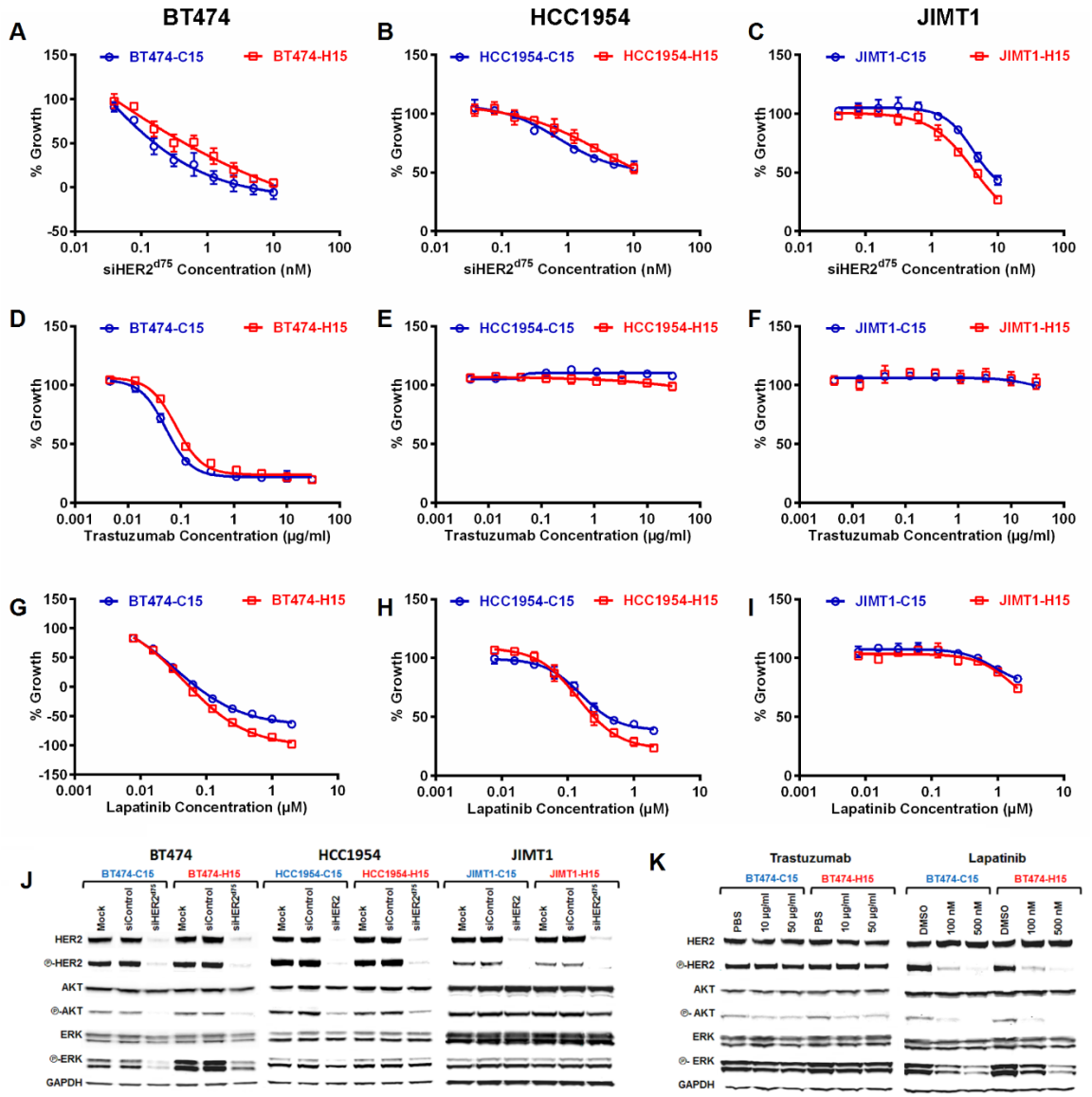

Figure 2.9 Growth inhibition and signaling pathways in long-term treated cells. Growth inhibitory effect (5 days) of (A – C) siHER2^{d75}, (D – F) trastuzumab, and (G – I) lapatinib in BT474, HCC1954, and JIMT1 cells that had been pre-treated weekly with 1 nM of siHER2^{d75} (H15) or siControl (C15) for 15 weeks. (J) HER2 signaling molecules in specified cell lines at 3 days after 10 nM siHER2^{d75} treatment. (K) HER2 signaling molecules in BT474-C15 and BT474-H15 at 3 days after 10-50 μg/ml trastuzumab or 100-500 nM lapatinib treatment.

Silencing delta16 HER2 with siHER2^{d75}

The delta16 HER2 is a more oncogenic variant of HER2 and has been implicated in tumor aggressiveness. Studies have shown that only 5 copies of delta16 HER2 are needed to drive oncogenic transformation, whereas 30-50 copies of WT HER2 were needed to initiate tumorigenesis (122). Delta16 HER2 has also been implicated in cancer resistance to trastuzumab (120) and tamoxifen (121). Therefore, the elimination of delta16 HER2 is of great therapeutic value. We hypothesize that since siHER2^{d75} targets a region upstream of exon 16, it should be able to silence both delta16 HER2 and WT HER2 with similar efficacy. Figure 2.10A – C shows the mRNA levels of wild-type HER2, delta16 HER2, and their ratios in parental BT474, BT474-TR, BT474-LR, and BT474-H15. A slight increase in the ratio of delta16 HER2 over WT HER2 was observed in BT474-TR, compared to that in parental BT474 ($p < 0.01$). In BT474-LR, the WT HER2 was upregulated, while delta16 HER2 was not. SiHER2^{d75} silenced both HER2 isoforms as shown by similar percent reduction in mRNA levels in BT474 cells at 48 h post-transfection (vs. siControl) (Figure 2.10D), confirming its broad specificity. This agrees with data from BT474-H15 (receiving a long-term treatment of siHER2^{d75}) that there is no significant change in delta16 HER2/WT HER2 ratio compared to the parental BT474 cells (Figure 2.10C).

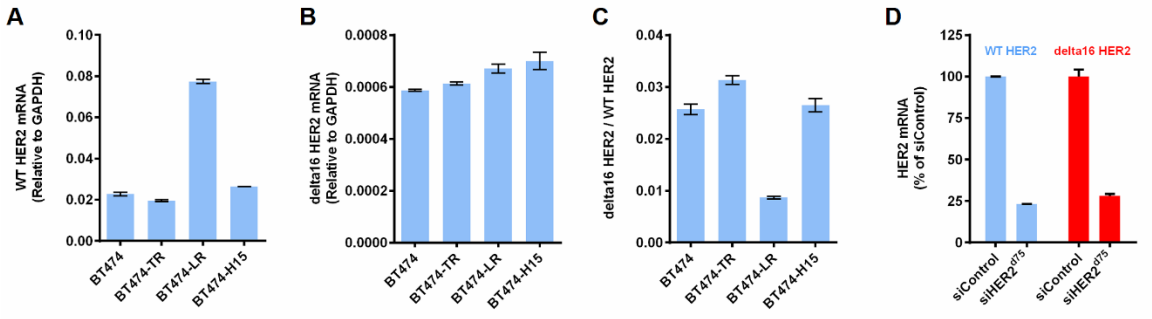

Figure 2.10 The siHER2^{d75} mediated gene knockdown of the delta16 HER2 splice variant. (A) WT HER2 mRNA levels of parental BT474, BT474-TR, BT474-LR, and BT474-H15. (B) Delta16 HER2 mRNA of corresponding cells. (C) Ratios of delta16 HER2 over WT HER2. (D) % Knockdown of WT HER2 mRNA and delta16 HER2 mRNA in BT474 cells at 48 h post transfection with 10 nM siHER2^{d75} (vs. siControl).

Delivery of siHER2^{d75} to HER2-positive breast cancer with targeted nanoconstructs

To show clinical and translational potential, we systemically delivered siHER2^{d75} to HER2-positive breast tumors with trastuzumab-conjugated mesoporous silica based nanoconstructs (designated “T-NP”) recently reported by us (253). The silica core has a uniform diameter of 50 nm and is further modified with the addition of layers of polyethylenimine (PEI), polyethyleneglycol (PEG), trastuzumab, and siRNA (Figure 2.11). The hydrodynamic size of the particles is 104 ± 1.7 nm and the zeta potential is 8.10 ± 0.3 mV in 10 nM NaCl after loading of siHER2^{d75}. When delivered with our nanoparticles, siHER2^{d75} triggered less or comparable immune response when compared to the FDA-approved nanoparticle based drugs Abraxane and Feraheme (253). The material given IV could treat

intrinsically resistant HCC1954 tumors (significantly after a couple doses) and knock down 60% of HER2 protein in the tumors after one dose (253).

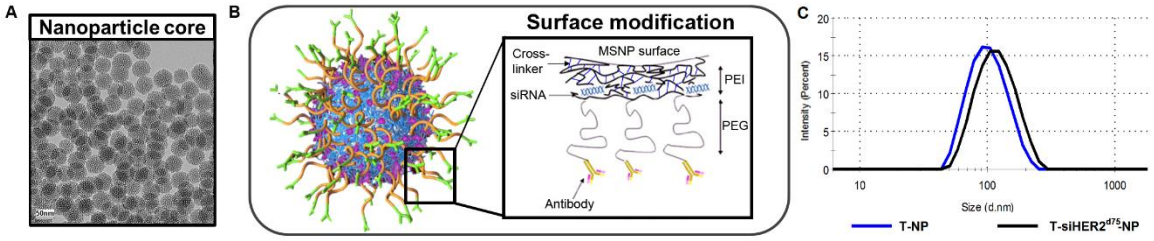

Figure 2.11 Trastuzumab-conjugated mesoporous silica nanoconstruct. (A) TEM image of the mesoporous silica nanoparticle (MSNP) core (scale bar = 50 nm). (B) Schematic of surface modification of MSNP (layer-by-layer) with cross-linked polyethylenimine (PEI), polyethyleneglycol (PEG), trastuzumab, and siRNA. (C) Hydrodynamic size distribution of T-NP (blue line) and with siRNA loading (black line).

Here we show that the T-siHER2^{d75}-NP could also treat a BT474 variant with acquired resistance to trastuzumab. The BT474 variant was developed in Kerbel's lab (259) by serial passaging BT474 in mice for 3 years. The resulting tumors grow aggressively in mice without estrogen supplement (unlike the parental BT474 tumors). After 2-6 months of trastuzumab treatment (20 mg/kg twice weekly, IP), the tumor that developed resistance was adapted into tissue culture. We subsequently passaged the cells in mice once more to further select for those that grew aggressively. These new tumors were adapted into culture and named BT474-TRgf. In addition to being selected with trastuzumab *in vivo*, the BT474-TRgf maintained its resistance to trastuzumab *in vitro* (Figure 2.12A). Figure 2.12B

shows that the BT474-TRgf cell grown in mice still responds well to our T-siHER2^{d75}-NP treatment, compared to the untreated or T-siSCR-NP. The tumors also showed a better response to our T-siHER2^{d75}-NP than free trastuzumab, given IV (twice weekly) at the equivalent dose to that of the nanoconstructs. The fact that tumors were not responsive to T-siSCR-NP also indicates that growth inhibition is due to siHER2, rather than trastuzumab on the nanoparticles.

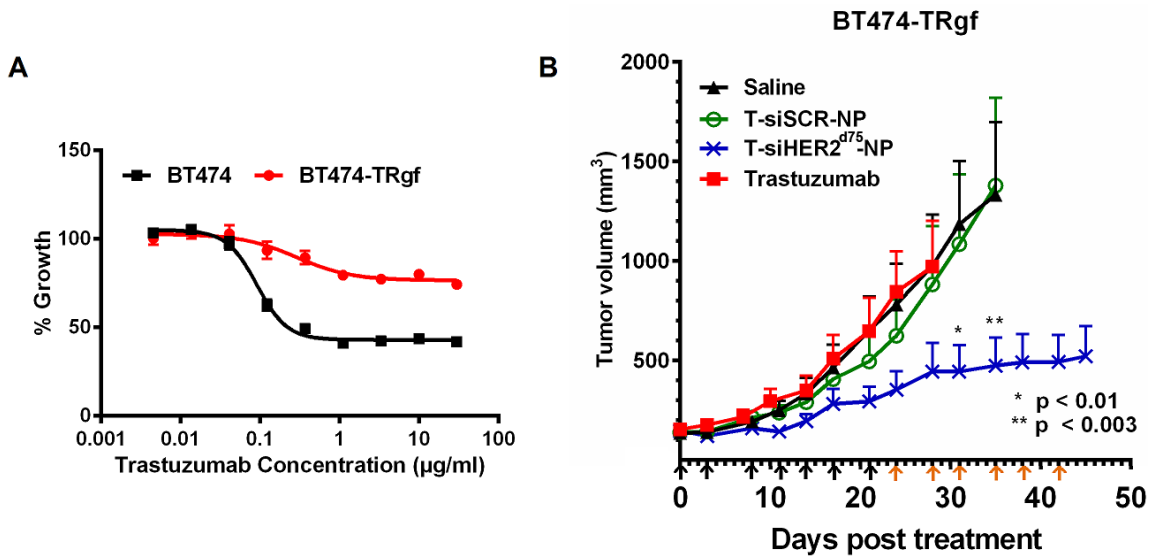

Figure 2.12 Efficacy of T-siHER2^{d75}-NP in BT474-TRgf. (A) *In vitro* trastuzumab dose-response curve (as % growth 5 days post-treatment) of BT474 and BT474-TRgf. (B) Mice bearing BT474-TRgf xenografts (n = 5 – 7/group) were injected via tail vein with saline, trastuzumab (2.5 mg/kg given IV, twice weekly) or trastuzumab-conjugated nanoparticles (T-NP) loaded with siHER2^{d75} or siSCR. Arrows below the x-axis indicate nanoparticles injections; black indicate 1.25 mg siRNA/kg; orange, 2.5 mg siRNA/kg. Tumor volumes are presented as means ± SEM. Specified p-values are against the saline control.

Cytotoxicity of T-siHER2^{d75}-NP in normal/non-tumorigenic HER2-negative cells

We evaluated the cytotoxicity of T-siHER2^{d75}-NP in normal/non-tumorigenic (HER2-negative) cells including HEK293 (human embryonic kidney cells), HEPG2 (human liver hepatocellular cells), MCF10A (human mammary epithelial cells), and HUVEC (human umbilical vein endothelial cells) as shown in Figure 2.13. Kidney and liver cell lines have been recommended by the Nanotechnology Characterization Lab (NCL) of NCI for cytotoxicity studies of nanoparticles since kidney and liver are clearance organs for nanoparticles (255, 260). MCF10A is considered a normal counterpart of breast cancer, and HUVEC is a relevant cell model for intravenous administration of nanoparticles. Figure 2.13A shows that these cells have no detectable HER2 expression when compared to BT474. At the efficacious dose for killing cancer cells (253), Figure 2.13B shows that our T-siHER2^{d75}-NP did not elicit significant cytotoxicity in any cell line tested (<10% cell death). T-siHER2^{d75}-NP appeared safer than the drug benchmark, APAP, recommended by NCL for nanoparticles' cytotoxicity study.

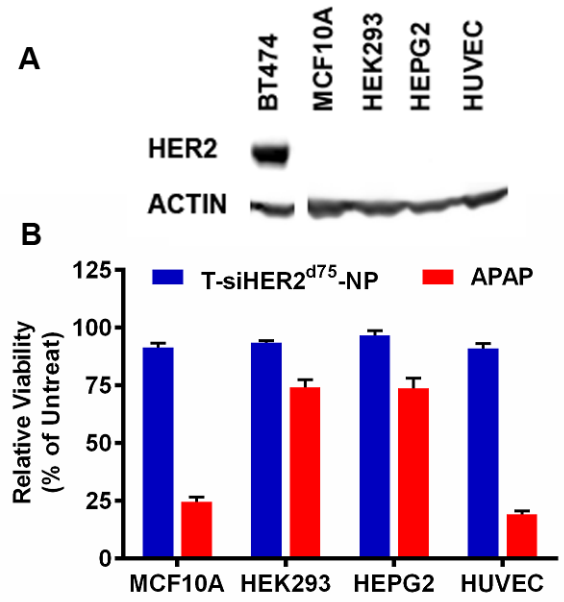

Figure 2.13 Cytotoxicity of T-siHER2^{d75}-NP in a panel of non-tumorigenic cell lines, MCF10A, HEK293, HEPG2, and HUVEC. (A) Western blots indicate no detectable HER2 expression in these cell lines when compared to BT474. (B) Low cytotoxicity (<10% cell death) of T-siHER2^{d75}-NP at efficacious dose (60 nM as siHER2^{d75}) compared to the drug benchmark, APAP (25 mM). Cell viability was measured at 24 h after the treatment and reported as the percentage of the untreated control.

Discussion

One major challenge today in treating HER2-positive cancer is the intrinsic and acquired resistance to HER2-targeted therapies. Studies to understand the intrinsic and acquired resistance to HER2-targeted therapies have implicated post-translational variants, splicing variants, and mutations of HER2 as potential mechanisms. One variant, p95-HER2, is a truncated form of HER2 that lacks the

extracellular domain thus rendering it incapable of binding trastuzumab (117). However, this form retains a functional HER2 kinase domain, and its level is enhanced in nodal metastatic tissues and is inversely correlated with disease free survival (116). Another HER2 variant is delta16 HER2, which lacks exon 16 (118). The absence of exon 16 exposes a cysteine residue, which promotes receptor homodimerization and activation (120). The exogenous overexpression of delta16 HER2, but not wild-type HER2, was sufficient to evoke an aggressive breast cancer phenotype in the otherwise non-tumorigenic MCF-10A mammary epithelial cell line (123). Clinically, delta16 HER2 expression was found in approximately 90% of HER2-positive breast cancer (data from 46 human HER2-positive BCs) and is highly correlated with dissemination to local lymph nodes (120). Delta16 HER2 has also been shown to activate entirely different signaling pathways than wild-type HER2 (123, 261), which may alter how cells respond to targeted therapies. A mutation in the kinase domain of HER2 also has been reported in cells (249) and in patient tissue samples (146), which may hinder the binding of lapatinib making it ineffective at blocking HER2 phosphorylation (249).

Other proteins have also been implicated in drug resistance of HER2-positive cancer. For instance, Wang et al. (94) reported that upregulation of estrogen receptors was primarily responsible for the initial resistance to both trastuzumab and lapatinib. However, in the later stage of resistance, cancer cells once again became reliant on HER2 signaling. Masking the HER2 receptor by a membrane-associated glycoprotein, MUC4, has been shown to prevent the binding of trastuzumab in HER2-positive JIMT1 cells (108). In addition, MUC4 (106)

and another family member MUC1-C (105), have also been shown to maintain HER2 phosphorylation in MCF7/HER2-18 (MCF7, engineered to overexpress HER2) and in BT474R and SKBR3R (resistant to trastuzumab after long-term selection) cells, respectively. Lastly, increased expression of HER2 receptor ligands that can stimulate HER2 activation, has been reported in a BT474 cell line with acquired resistance to lapatinib (94).

These mechanistic studies suggest that resistant cancers still depend on HER2, while highlighting the shortcomings of monoclonal antibodies and small molecule inhibitors targeting HER2 in the resistant cells. Herein, we show that RNA interference (RNAi) by siRNA, which halts protein synthesis at the mRNA level, can potentially overcome these shortcomings. Moreover, siRNA can be designed to target mutated HER2 or HER2 variants if needed.

Our success in delivering effective doses of siHER2^{d75} to treat intrinsically resistant HCC1954 tumors (253) prompted us to test it in a model of HER2-positive cancer having acquired resistance to trastuzumab (BT474-TR) and lapatinib (BT474-LR). For HER2 siRNA screening *in vitro*, we used a non-specific commercial transfection agent instead of our nanoparticle delivery platform to avoid confounding siHER2 effects with those of trastuzumab, which is used as the homing target agent on our nanoparticles. Our resistant BT474-TR and BT474-LR behaved similarly to those previously reported by Wang et al. (94); their growth was not fully inhibited by exceedingly high doses of trastuzumab and lapatinib, respectively. In contrast, siHER2^{d75} was effective in inhibiting growth of both

BT474-TR and BT474-LR cells. The results translated well to the trastuzumab-resistant BT474-TRgf tumor xenografts.

In our study, we showed that cancer treated long-term with siHER2^{d75} remained sensitive to the siHER2, lapatinib and trastuzumab without significant changes in the downstream phosphorylation patterns. This suggests that HER2 signaling is still the preferred signaling pathway and that the cells remained addicted to HER2-mediated survival and proliferation. Cancer is less prone to develop acquired resistance to siHER2^{d75} treatment because it circumvents many resistance mechanisms reported for small molecule inhibitors or antibodies. Specifically, siHER2^{d75} decreases HER2 mRNA, and therefore the cells are unable to synthesize more HER2 proteins to compensate for the loss of HER2 through receptor recycling and degradation. Removing HER2 by siHER2^{d75} can also negate resistance mechanisms relying on the presence or stimulation of HER2 proteins as aforementioned. In addition, siHER2^{d75} is not susceptible to resistance caused by mutations in the kinase domain or truncation of extracellular domain of HER2 that could otherwise prevent the binding of trastuzumab and lapatinib.

In all, our results suggest that siHER2^{d75} is a promising alternative to current HER2-targeted therapies. Moreover, HER2-positive tumors that have progressed on trastuzumab or lapatinib are likely to remain treatable with siRNA against HER2 while there is minimal risk of developing further resistance to this RNAi strategy. Thus, the clinical translation of HER2 siRNA to treat resistant HER2-positive cancer is highly viable.

CHAPTER 3

EFFECT OF LONG-TERM HER2 SIRNA TREATMENT

Abstract

Intrinsic and acquired resistance to current HER2 targeted therapies remains a challenge in clinics. We have developed a siRNA based therapeutic against HER2 that is delivered using mesoporous silica nanoparticles modified with polymers and conjugated with HER2 targeting antibodies. Our previous studies have shown that our HER2 siRNA nanoparticles could overcome intrinsic and acquired resistance to trastuzumab and lapatinib in HER2-positive breast cancers. In this study, we investigated the effect of long-term (7 months) treatment using our therapeutic HER2 siRNA. Even after the removal of HER2 siRNA, the long-term treated cells grew much slower (67% increase in doubling time) than naïve cells. Furthermore, the level of tumor initiating cells remained unchanged and showed no indication of an epithelial-mesenchymal transition. Unlike trastuzumab and lapatinib, which induced resistance in BT474 cells after 6 months of treatment, HER2 siRNA did not induce resistance to HER2 siRNA, trastuzumab, or lapatinib. HER2 ablation with HER2 siRNA prevented reactivation of HER2 signaling that is observed in cells resistant to lapatinib. Altogether, our results indicate that a HER2 siRNA based therapeutic provides a more durable inhibition of HER2 signaling and is more effective than existing therapeutic monoclonal antibodies and small molecule inhibitors.

Introduction

Overexpression or amplification of HER2 (*ERBB2*) occurs in several types of cancer including breast (262), ovarian (263, 264), gastric (265) and colorectal cancers (266, 267). In breast cancer, approximately 20% of all cases fall into the HER2+ subtype, which is an adverse prognosis factor (242). Current therapeutic regimens include the use of monoclonal antibodies or small molecule inhibitors in combination with a chemotherapy such as docetaxel (or other taxane). Approved in 1998, the humanized monoclonal antibody trastuzumab binds to domain IV of HER2 and blocks receptor homodimerization (268). To complement the activity of trastuzumab, another monoclonal antibody, pertuzumab, was developed to bind domain II of HER2 to block heterodimerization with other HER family receptors (40). Following positive results of the CLEOPATRA trial, these two antibodies are now used with docetaxel as first-line treatment for metastatic HER2+ breast cancer (42, 269). The recently approved trastuzumab emtansine (T-DM1), trastuzumab conjugated to the microtubule inhibitor emtansine, is designated as a second-line treatment (47). Lapatinib, a small molecule inhibitor that binds to the kinase domain of HER2 and EGFR (247, 270), is used in combination with trastuzumab or capecitabine as third-line treatment (271).

Despite the development and use of HER2 targeted therapeutics, intrinsic and acquired resistance remains a challenge in the clinics. For instance, in the phase 3 MARIANNE study, 67.9% of patients responded to trastuzumab plus a taxane with a median response duration of 12.5 months, while 64.2% of patients responded to T-DM1 plus pertuzumab with a median response of 21.2 months (49).

Ongoing research into the mechanisms of resistance have revealed several molecular adaptations in which tumor cells can compensate for or circumvent the inhibition of HER2 signaling. Mutations in the kinase domain of HER2 that cause resistance to lapatinib have been characterized but are rare (146, 148, 249). Truncation of the extracellular domain of HER2 produces a variant called p95HER2 that does not respond to monoclonal antibodies (115-117). Upregulation of MUC4 can block binding of trastuzumab (108, 109). There have been reports of Δ16HER2 in which exon 16 is excised during splicing, resulting in a form that homodimerizes more readily (120, 124). In addition, cancer cells can overcome HER2 blockade by signaling through other HER family receptors (91, 92, 272) or receptor crosstalk via MUC1-C (105), IGFR (98), MET (103) and FGFR (100). Furthermore, studies have shown that resistant cancer cells can reactivate the HER2 pathway via secretion of HER receptor ligands, and thus remain dependent on HER2 signaling (89, 94).

We sought to find an alternative therapeutic approach that would provide a durable response in contrast to the current propensity for developing resistance to monoclonal antibodies and small molecule inhibitors. We developed a pre-clinical HER2 siRNA based therapeutic delivered using functionalized mesoporous silica nanoparticles. The nanoparticles consisted of 50-nm silica cores coated with a cationic polymer and PEG, and are conjugated with trastuzumab for HER2 targeting. We have previously shown that our HER2 siRNA nanotherapeutic has an excellent safety profile and could overcome intrinsic and acquired resistance to trastuzumab and lapatinib in HER2-positive breast cancers *in vitro* and *in vivo* (273,

274). In the current study, we investigate the response duration of cancer cells treated with HER2 siRNA delivered by our nanoparticles and the commercial transfection reagent DharmaFECT as the benchmark. While DharmaFECT can be used in vitro to study the functional significance of long-term HER2 ablation, the ability of our nanoparticles to deliver siRNA *in vivo* has far more clinical relevance. We hypothesized that ablation of HER2 protein by siRNA can prevent the rapid onset of resistance. We compared how the cells differ in their response to trastuzumab, lapatinib or HER2 siRNA after long-term treatment to these drugs. We also explored changes in protein expression and phosphorylation using reverse phase protein arrays (RPPA) to determine the adaptive changes necessary to survive in a low HER2 environment. In all, our findings suggest that targeting HER2+ cancer using siRNA is more durable and effective than monoclonal antibodies or small molecule inhibitors.

Materials and methods

Synthesis of nanoparticles and preparation of siRNA complexes

Mesoporous silica nanoparticles modified with polymer and conjugated to trastuzumab were synthesized and characterized as previously reported (239). HER2 and non-targeting control siRNAs (siSCR) purchased from Dharmacon™ were loaded onto nanoparticles at 2 wt.% prior to transfection. For transfection using Dharmafect, siRNAs were diluted in OptiMEM medium and a final dilution ratio of 1:200 was used for Dharmafect.

Cell culture and long-term treatment

BT474 was obtained from ATCC and maintained in RPMI1640 growth medium supplemented with 10% fetal bovine serum. BT474-TR and BT474-LR were generated by growing the parental BT474 under increasing concentrations of trastuzumab or lapatinib for 6 months, as previously reported (273). For long-term siRNA transfection, cells were seeded in 6-well plates and transfected weekly with 60 nM HER2 siRNA or 60 nM scrambled siRNA delivered by nanoparticles or DharmaFECT.

Cell viability assay

Cell viability was determined using the CellTiter-Glo assay (Promega). Cells were seeded in 96-well plates and allowed to attach for 24 h prior to transfection or drug treatment. Plates were read 3 days after lapatinib treatment or 5 days after trastuzumab treatment or siRNA transfection. For treatment with siRNAs, cell media was changed 24 hours after transfection.

Flow cytometry

Freshly harvested cells were washed in FACS buffer (pH 7.4 PBS with 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1% FBS and 0.02% azide) and aliquoted into 1×10^6 fractions for staining. CD24-FITC and CD44-APC antibodies (BD Biosciences) were added according to the manufacturer's recommended dilution and the samples were incubated on ice with shaking for 30 min. After two washes, samples were resuspended in 500 µl of FACS buffer and read on a Millipore Guava

easyCyte 12 flow cytometer. HER2 was stained using 1 µg of trastuzumab per sample followed by washing and incubation with 1 µg of Alexa 647 conjugated anti-human secondary antibody.

Western blot

Cells were lysed in RIPA buffer, sonicated and protein was quantified using BCA assay. After adding 4X Novex NuPAGE LDS sample and 10% beta-mercaptoethanol (BME), the samples were denatured for 5 min at 95 °C. Twenty to 30 µg of proteins were loaded per lane onto 4 – 12% Bis-Tris NuPAGE gels. Following gel electrophoresis, proteins were transferred onto PVDF-FL membrane, blocked with LI-COR blocking buffer and incubated with primary antibodies overnight at 4 °C. IRDye conjugated secondary antibodies were added the next day and membranes were scanned on a LI-COR Odyssey CLx imaging system. Band densitometry was analyzed using ImageJ.

Reverse phase protein microarray

Proteins from the parental and long-term treated BT474 derivatives were harvested and processed according to the protocol from MD Anderson's RPPA core facility website (https://www.mdanderson.org/documents/core-facilities/Functional%20Proteomics%20RPPA%20Core%20Facility/RPPA_Frozen%20Tissue%20Lysate%20Prep_Precellys%20Homogenizer.pdf). Briefly, cells in 6-well plates were lysed and proteins were collected. Debris was removed after centrifugation and proteins were quantified and adjusted to 1.5 µg/µl. SDS and

BME were added and samples were denatured at 96 °C for 5 min. Protein samples were stored at -80 °C until dispatched to MD Anderson's RPPA core facility for analysis. Detailed RPPA process and methods can be found at https://www.mdanderson.org/content/dam/mdanderson/documents/core-facilities/Functional%20Proteomics%20RPPA%20Core%20Facility/RPPA%20Materials_Methods_2016.pdf.

Statistical analysis

Experiments were performed in three or more replicates with results reported as mean ± standard deviation (SD). The Student t test (normal distribution) or Mann-Whitney test (nonparametric, unpaired) was used for group comparisons. Multiple comparisons of three or more groups were done using one-way ANOVA (normal distribution) or the Kruskal-Wallis nonparametric test with post-hoc Dunnett multiple comparison tests. GraphPad Prism 6.0 software (GraphPad Software Inc.) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

Results

We previously reported that BT474 cells became resistant to 10 μg/ml of trastuzumab or 1 μM of lapatinib within 6 months of continued treatment (273). To determine whether the BT474 cells could develop resistance to HER2 siRNA, we performed similar long-term treatment with weekly transfections of HER2 siRNA delivered with either DharmaFECT or our mesoporous silica nanoparticles for 30

weeks. Nanoparticles with (TNP) and without trastuzumab (NP) were tested in order to account for any therapeutic effect of trastuzumab. Trastuzumab conjugated nanoparticles showed uptake specificity to HER2-positive cancer cells over HER2-negative cells after a short contact time of 30 minutes to 2 hours [36], while nanoparticles without trastuzumab (more positively charged) enter the cells in a non-specific manner after long exposure. Separate populations were treated with scrambled siRNAs (siSCR) for the same duration as another control. The resulting derivatives and treatment description are listed in Table 3.1, and their names will be used thereafter.

Table 3.1 Name and corresponding treatment of BT474 derivatives

Name	Treatment	Description
BT474-C30	siSCR	scrambled siRNA with DharmaFECT
BT474-H30	siHER2	HER2 siRNA with DharmaFECT
BT474-NP-C30	siSCR-NP	scrambled siRNA on nanoparticles
BT474-NP-H30	siHER2-NP	HER2 siRNA on nanoparticles
BT474-TNP-C30	T-siSCR-NP	scrambled siRNA on trastuzumab conjugated nanoparticles
BT474-TNP-H30	T-siHER2-NP	HER2 siRNA on trastuzumab conjugated nanoparticles
BT474-TR	Trastuzumab	Trastuzumab resistant BT474
BT474-LR	Lapatinib	Lapatinib resistant BT474

Reduced cell cluster size and growth rate after long-term treatment with HER2 siRNA on nanoparticles conjugated to trastuzumab

Figure 3.1A – C shows the cell morphology and growth rate for each of the BT474 derivatives following the long-term treatment. Cells were seeded at the same density 5 days before imaging or nuclei counting. BT474 treated with HER2 siRNA on nanoparticles conjugated with trastuzumab grew 30% slower than the

parental or the scrambled siRNA treated counterparts (Figure 3.1B) with a doubling time that was almost twice as long (Figure 3.1C). Interestingly, we did not observe such change in cells treated with HER2 siRNA or scrambled siRNA delivered using DharmaFECT or nanoparticle without trastuzumab. This may owe to increased cellular uptake and/or a therapeutic effect of trastuzumab in addition to that of HER2 siRNA.

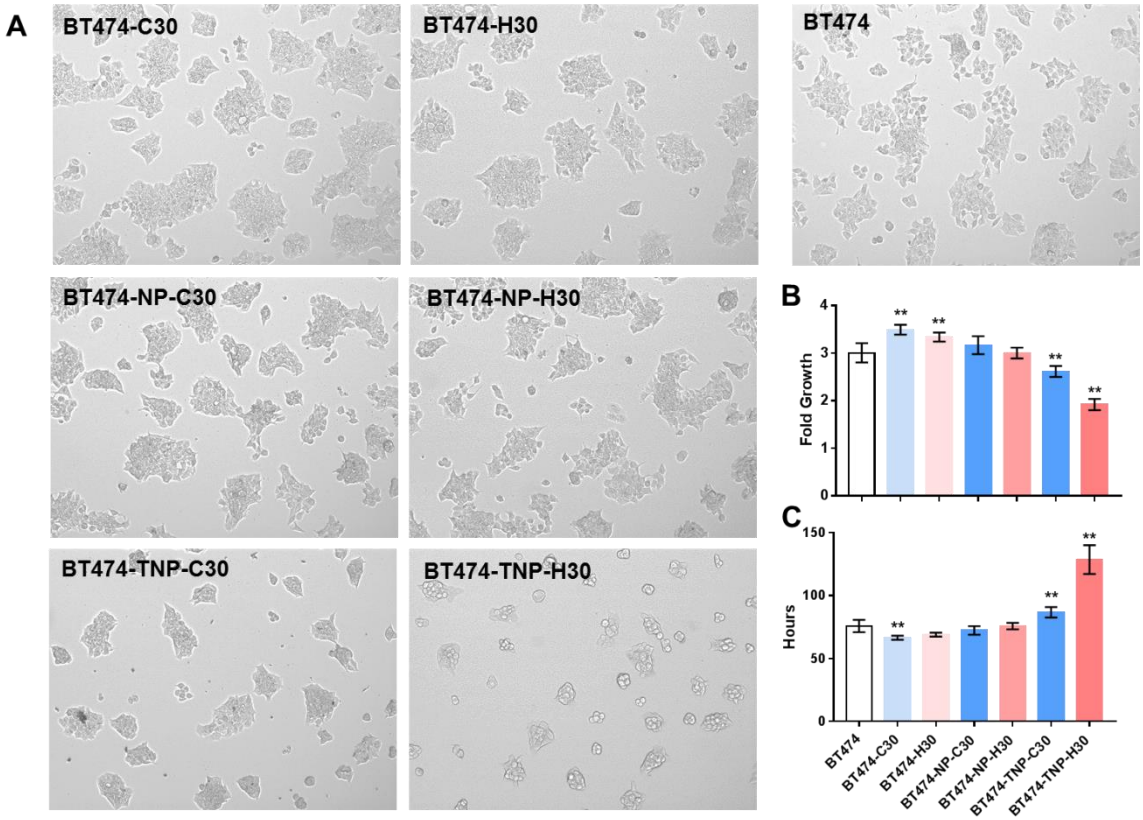

Figure 3.1 Phenotype of long-term siRNA treated cells. When seeded at the same density for 5 days without treatment, BT474-TNP-H30 grew 30% slower than the parental BT474 or other derivatives. Images showing representative fields at 100X magnification (A). Fold change in nuclei count after 5 days of growth (B) and the corresponding doubling time (C). Bars represent mean ± SD of 6 replicates in

a 96-well plate. Double asterisks ** indicate statistical significance when compared to the parental BT474 ($P < 0.05$).

Long-term HER2 siRNA treatment did not lead to epithelial-mesenchymal transition or tumor initiating cell enrichment

One mechanism by which cancer cells can develop drug resistance is through an epithelial to mesenchymal transition (EMT). Cells undergoing EMT typically lose cell adhesion and become more motile, which are precursors to metastasis (275). Cells undergoing EMT would have a decrease in the expression of E-cadherin and an increase in the expression of vimentin. Western blots of cell lysates showed that there was no concurrent downregulation of E-cadherin and upregulation of vimentin among the BT474 derivatives (Figure 3.2A). Next, we investigated the surface expression of CD24 and CD44 by flow cytometry, in which the CD24-/CD44+ population is indicative of potential tumor initiating cells with invasive and drug resistance capabilities (276). The parental BT474 cells were almost exclusively all CD24+/CD44-, which is characteristic of a luminal breast cancer phenotype. Following long-term HER2 siRNA treatment, the population distribution remains largely the same, without any detectable emergence of a CD24-/CD44+ population (Figure 3.2B), suggesting no enrichment of tumor initiating cells.

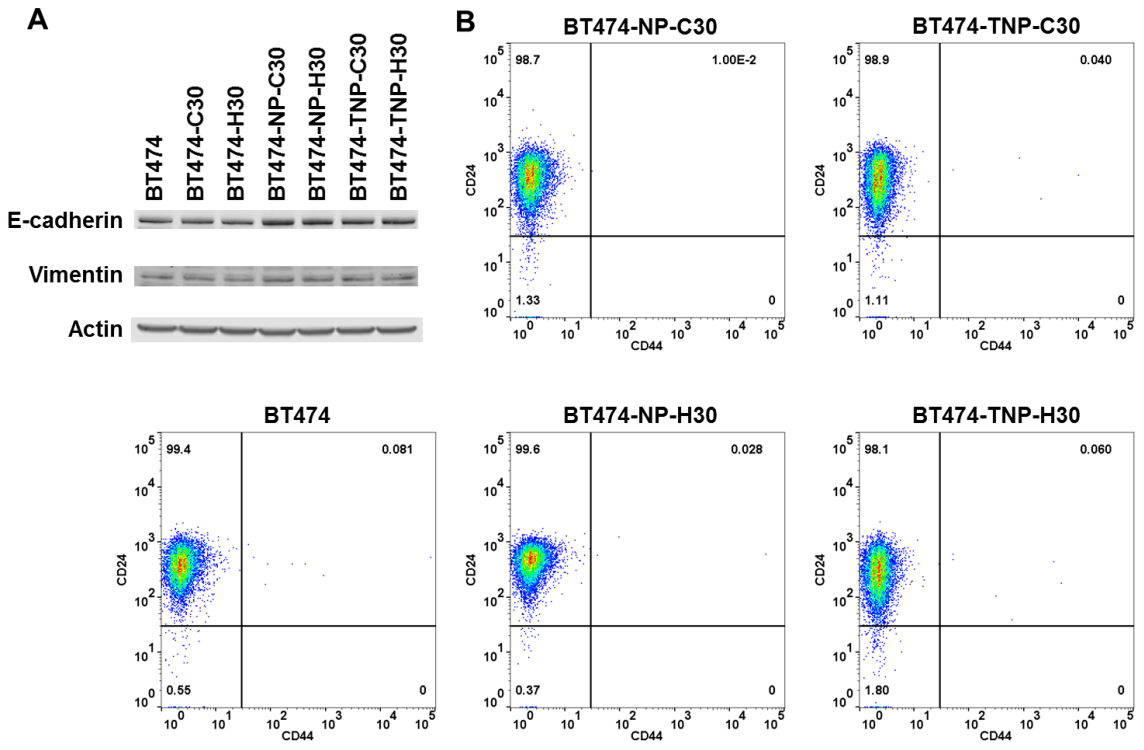

Figure 3.2 EMT and TIC characteristics of long-term HER2 siRNA treated cells. Western blot showed no concurrent downregulation of E-cadherin and upregulation of vimentin in BT474 derivatives compared to parental BT474, indicating no evidence of epithelial to mesenchymal transition (A). Flow cytometry was used to determine the surface expression of CD24 and CD44 among the BT474 derivatives. There was no enrichment of tumor initiating cells (CD24-/CD44+) in the long-term treated cells.

Long-term HER2 siRNA treated cells remained sensitive to HER2 siRNA, trastuzumab and lapatinib

We proceeded to challenge the BT474 derivatives with the same materials that they received during the 30-week treatment and assessed their cell death response. The response of BT474-H30 was nearly identical to that of BT474-C30

or parental cells (Figure 3.3A), while a reduced response was observed in those treated with HER2 siRNA delivered with nanoparticles with (T-siHER2-NP) or without trastuzumab (siHER2-NP) (Figure 3.3B – C). For T-siHER2-NP treated cells, the reduced response was not observed at the 8 week mark, but appeared after 15 weeks (Figure 3.4) and remained the same from that point on. For siHER2-NP treated cells, the reduced response was not observed at 15 weeks (Figure 3.4), but appeared at 30 weeks. The greater reduction in response in the T-siHER2-NP group may be partially explained by phenotypic change (slower growth rate) shown in Figure 4.1.

We then evaluated the response of BT474 treated for 7 months (30 weeks) with HER2 siRNA in comparison to those treated for 6 months with trastuzumab and lapatinib, BT474-TR (Figure 3.3D – F), and BT474-LR (Figure 3.3G – I), respectively. While BT474-TR and BT474-LR were resistant to trastuzumab and lapatinib, respectively, all cells treated long-term with HER2 siRNA (delivered by DharmaFECT, NP, or TNP) did not show resistance to either drug. Furthermore, while cells become resistance to long-term treatment of free trastuzumab, they were not resistant to trastuzumab on the nanoparticles (see BT474-TR vs. BT-474-TNP-C30, Figure 3.3F). In short, there was minimal change in terms of how long-term HER2 siRNA treated cells responded to renewed HER2 silencing or inhibition using lapatinib or trastuzumab. Thus, silencing HER2 did not induce adaptive responses that would have rendered the cells resistant to repeated HER2 silencing (with siRNA) or inhibition, at least not to the same degree and same time frame as in BT474-LR or BT474-TR.

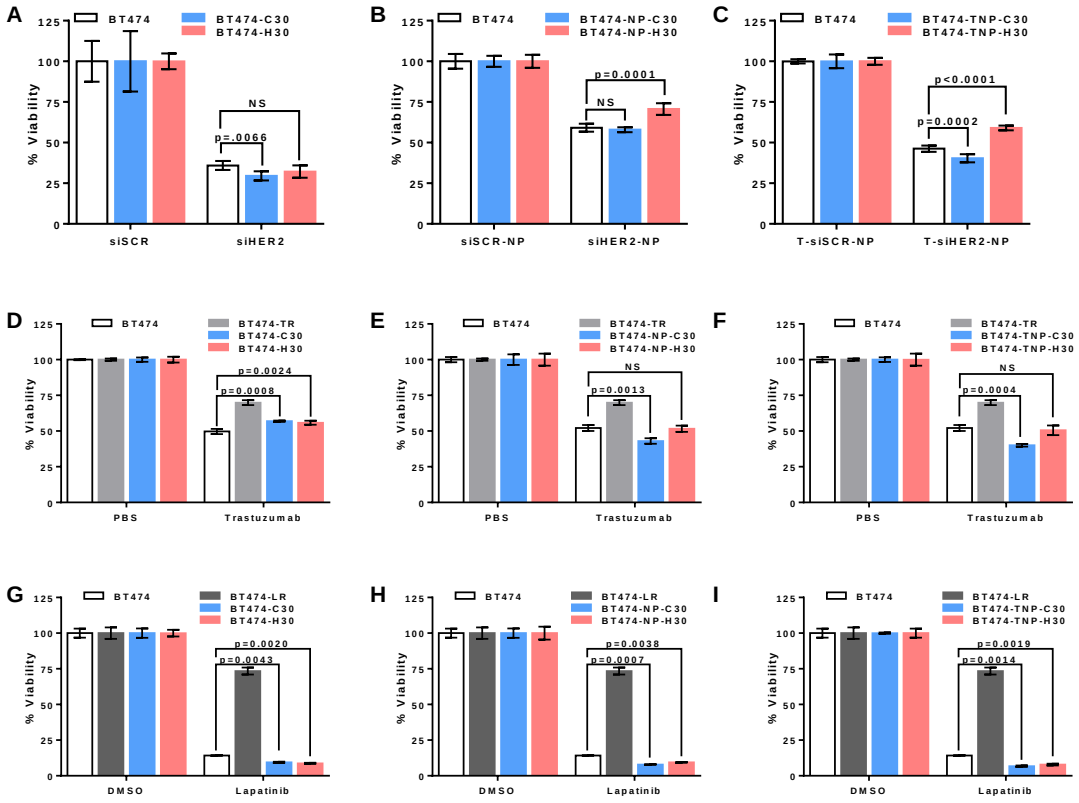

Figure 3.3 Cell viability of BT474 derivatives when challenged with their respective long-term treatment regimens. Long-term siHER2 treated BT474 derivatives were challenged with HER2 siRNA delivered using either DharmaFECT (A), nanoparticles (NP) (B), or trastuzumab conjugated nanoparticles (TNP) (C). Their response to trastuzumab (D – F) or lapatinib (G – I) were compared those of the resistant derivatives BT474-TR and BT474-LR.

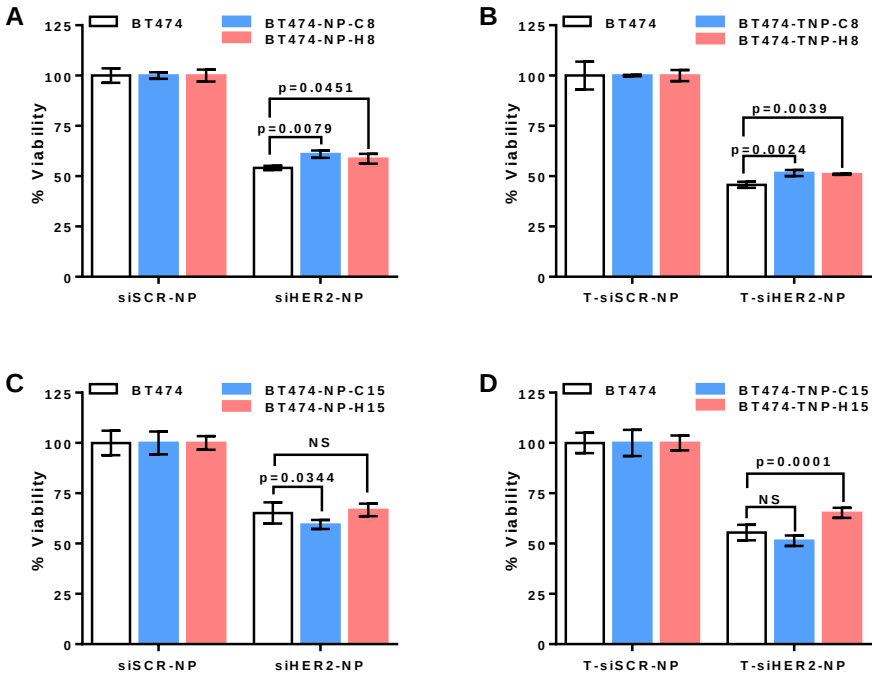

Figure 3.4 Cell viability of BT474 derivatives at earlier time points during long-term HER2 siRNA treatment. BT474 derivatives were challenged with HER2 siRNA delivered using nanoparticles (A and C), or trastuzumab conjugated nanoparticles (B and D) after 8 weeks (A and B) or 15 weeks (C and D) of treatment.

HER2 ablation prevents reactivation of HER2 signaling

To explore how HER2 siRNA treated cells differ in adaptation to the selective environment when compared to BT474-TR and BT474-LR, we looked at changes in protein expression and phosphorylation using reverse phase protein arrays. Data are shown in Figure 5. The parental BT474 served as a baseline (no treatment). BT474-TR, BT474-LR, BT474-TNP-H30 and BT474-TNP-C30 were sampled while under treatment of trastuzumab, lapatinib, HER2 siRNA and scrambled siRNA delivered with TNP, respectively. In lapatinib resistant BT474-

LR, we observed an increase in the expression of ER and HER2, in agreement with compensation through ER and reactivation of HER2 signaling in lapatinib resistant cells reported by Wang and colleagues (94). Phosphorylation of AKT, ERK and S6 ribosomal protein were sustained even in the presence of lapatinib. There was a slight increase in the expression and phosphorylation of Rictor, which is a cofactor of the mTOR complex 2 that was shown to mediate resistance through phosphorylation of AKT on serine 473 (277). Phosphorylation of focal adhesion kinase (FAK) was also increased in BT474-LR and BT474-TR, which is consistent with a previous report of its physical association with HER2 to mediate receptor clustering and crosstalk (278). Upregulation of SLC1A5 (ASCT2), an alanine, serine, cysteine-preferring transporter 2 was observed in BT474-LR. This protein was shown to regulate glutamine uptake and promote growth of triple negative breast cancer by supporting the mTORC1 signaling (279). These changes, however, were not present in BT474-TNP-H30, in which reduced level of HER2 signaling was observed. BT474-TNP-C30 (with scrambled siRNA) also had downregulation of HER2, which can be attributed to trastuzumab on the particles. This suggests that even at very low dose (equivalent to 1 μg/ml trastuzumab), trastuzumab on the nanoparticles could reduce HER2 protein level via receptor internalization (along with the nanoparticles) and degradation. As a result, we did not observe higher levels of phospho-FAK in BT474-TNP-C30 as we did in BT474-TR.

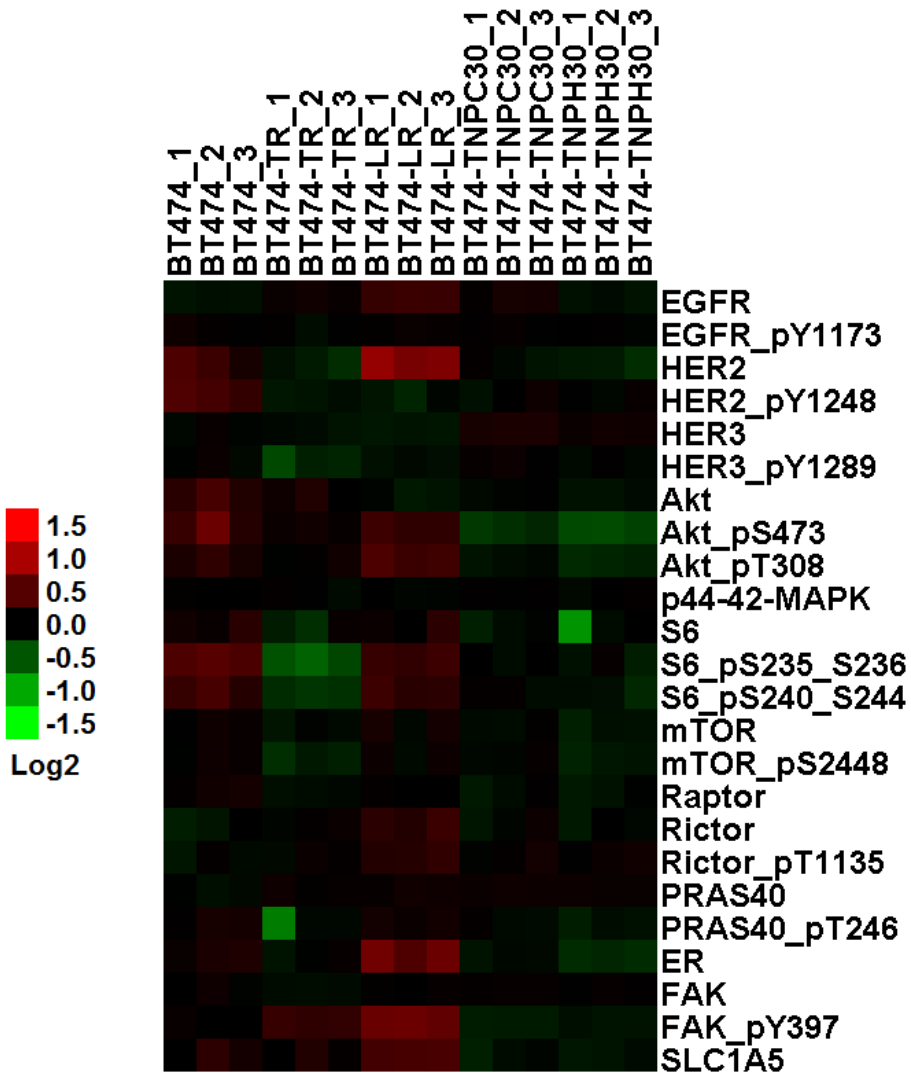

Figure 3.5 Heat map of selected genes that were differentially expressed as determined by RPPA. Proteins from BT474 derivatives while under their corresponding long-term treatment agents were spotted onto reverse phase protein microarrays and probed using a panel of antibodies. Data are presented in triplicate, with each data point representing the expression of a given protein from a single replicate. Color gradient represents protein expression levels (log base 2).

Discussion

Targeting the HER2 receptor using conventional monoclonal antibodies and small molecule inhibitors works by blocking dimerization and activation of the receptor. However, HER2 can bind many different receptors outside of the HER family and thus circumvent inhibition via receptor crosstalk and alternative signaling pathways. These adaptive changes can lead to the survival of a small population of cancer cells that persist throughout treatment, leading to the relapse of the disease. We showed that using siRNA to silence HER2 at the mRNA level, thereby halting synthesis of the HER2 protein, could prevent the aforementioned survival mechanisms.

In this study, we compared the endpoint of separate populations of HER2-positive BT474 cells after receiving trastuzumab, lapatinib or HER2 siRNA for 6 – 7 months. In the trastuzumab or lapatinib groups, the cells became markedly resistant, with a 250 and 20 fold increase in GI50 values for each, respectively, as reported previously (273). HER2 siRNA treated groups, on the other hand, remained sensitive to repeated HER2 silencing, trastuzumab and lapatinib. We did not observe resistance to long-term HER2 siRNA treatment when delivered with DharmaFECT, but 10% reduction in response when delivered with nanoparticles, especially those conjugated with trastuzumab. The reduction in the response could be attributed to the slower growth rate after T-siHER2-NP treatment. It is also important to note that in order to sustain long-term treatment, a low concentration of HER2 siRNA was required due to the observed inability to retain enough cells to subculture at increased concentrations. This further supports the conclusions

that HER2 siRNA treatment was highly growth inhibitory and that the BT474 cells were unable to circumvent siRNA mediated HER2 ablation. Our data also indicated that HER2 siRNA delivered by our nanoparticles did not induce an epithelial-mesenchymal transition or the enrichment of tumor initiating cells.

Reactivation of HER2 signaling after long-term exposure to lapatinib was attributed to the secretion of HER family receptor ligands as well as the HER2 L755S mutation (280). In our version of lapatinib resistant BT474-LR, we also observed higher expression of HER2 and ER. This derivative also displayed higher phosphorylation of focal adhesion kinase (FAK), which was implicated in HER2 receptor clustering with integrin β1, leading to amplified signaling (278). Another group has shown that inhibiting FAK could improve response to trastuzumab (281). These findings suggest that HER2-positive cancer cells can develop converging mechanisms to maintain HER2 signaling, and are therefore susceptible to HER2 ablation using siRNA.

While HER2 mutations are relatively rare in the clinic, their occurrence can effectively limit the efficacy of inhibitors or antibodies, as in the case of the L755S mutation. One major advantage of using siRNA as a therapeutic on a modular delivery platform such as our nanoparticles is that siRNA can be quickly redesigned and validated should a mutation render the original version ineffective, although new clinical trials would still be required.

Another benefit of siRNA therapeutics is the ability to target genes for which there are significant technical challenges in developing an effective small molecule inhibitor, as in the case of KRAS. A recently completed phase 1 trial delivered

KRAS siRNA intratumorally in combination with chemotherapy in advanced pancreatic cancer (282). The regimen was well-tolerated and showed promising efficacy, prompting the initiation of a phase 2 trial.

Systemic delivery of siRNA requires effective nanoparticle carriers to achieve promising response in tumors not amendable for local delivery. Our nanoparticle platform has been highly optimized and validated in various mouse models of human cancers (239, 273, 283), and was geared toward clinical trials. In this study, we reported for the first time the effect of long-term siRNA treatment in cancer. We showed that HER2-positive cancer cells are less likely to develop resistance to HER2 siRNA than to trastuzumab or lapatinib over the same duration of treatment. From a mechanistic standpoint, the ablation of HER2 protein is more effective than blocking HER2 activation. Therefore, our siRNA therapeutic has promising clinical values and warrants further clinical investigation.

CHAPTER 4

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Summary

In this dissertation, I investigated the feasibility and advantages of a nanoparticle-based siRNA therapeutic for targeting HER2+ breast cancer. Chapter 1 reviewed background literature on the biology of the HER2 oncogene, existing therapeutics and the mechanisms of resistance to these therapeutics. HER2 is a receptor tyrosine kinase that is amplified and overexpress in about 20% of breast cancer. High level of HER2 is associated with poor clinical outcomes because HER2 signaling promotes proliferation and survival. Activation of HER2 can occur in a ligand dependent fashion when it heterodimerizes with EGFR, HER3 or HER4. HER2 preferentially forms heterodimer with HER3, which has an inactive kinase domain. The HER2/HER3 heterodimer is a highly potent oncogenic driver. In the absence of ligand, HER2 can homodimerize. The truncated form, p95HER2, and the exon 16 skipped variant, Δ16HER2, can be especially potent in promoting the homodimerization of HER2. Signaling downstream of HER2 is mediated mainly through the RAS-MAPK and PI3K-AKT pathways.

The development of targeted therapies has significantly improved management of HER2+ breast cancer. The addition of the monoclonal antibodies trastuzumab and pertuzumab to chemotherapy can improve outcome in both metastatic and early HER2+ breast cancer. The binding of monoclonal antibodies blocks the homodimerization and heterodimerization of HER2 and triggers an

innate immune response, termed antibody dependent cell mediated cytotoxicity. Small molecule inhibitors such as lapatinib and neratinib target the kinase domain of HER2 and other HER family receptors. An antibody-drug conjugate, T-DM1, links the cytotoxic agent emtansine to trastuzumab and disrupts microtubule assembly. However, a significant number of patients do no respond to these therapies or will eventually develop resistance. While the antibody-drug conjugate T-DM1 can partially overcome resistance to trastuzumab, they themselves are vulnerable to the intrinsic and acquired of resistance (46).

Mechanisms of resistance to HER2 targeted therapy include compensation by signaling through other HER family receptors, receptor cross talk, decoupling of downstream signaling pathway, mutations and attenuation of innate immune response. In the presence of trastuzumab, which blocks HER2 homodimerization, cancer cells can increase the formation of EGFR/HER2 or HER2/HER3 heterodimers. When stimulated with the HER3 ligand neuregulin, the HER2/HER3 heterodimer can adopt a conformation that is refractory to lapatinib. Nuclear localization of HER4 has also been reported to mediate trastuzumab resistance. In resistant cancer cells, HER2 has been found to associate with other non-canonical binding partner such as IGFR and MUC1-C, which can enhance and maintain HER2 phosphorylation. Upregulation of FGFR, MET and AXL have been found to confer survival when treated with trastuzumab or lapatinib. In hormone receptor positive HER2+ breast cancer, signaling through ER can promote survival in the early state of resistance. PIK3CA mutation and PTEN loss have been associated with trastuzumab resistance, but recent analysis of clinical results

showed no definitive correlation and therefore the association remains debatable. A number of somatic mutations in the HER2 receptor confer lapatinib resistance. Finally, trastuzumab response appears to correlate with the patient's immune response, where expression of pro-immune genes and tumor infiltrating lymphocytes were associated with better outcome.

Chapter 1 also reviewed the mechanism of RNA interference, the different types of synthetic RNA to trigger the RNAi pathway, and various classes of nanoparticles as delivery vehicle. RNAi is a mechanism of gene attenuation by which a short RNA duplex triggers the degradation of the messenger RNA or translational repression. SiRNAs are 19 – 23 nucleotide RNA molecules with 3' overhangs. When delivered to cells, they are loaded onto the RNA induced silencing complex, which unwinds the molecule and retains the strand that is complementary to the target mRNA. The endonuclease Argonaute 2 can then cleave the target mRNA. In contrast to microRNAs, which can target a number of genes at once, siRNAs are highly specific and are therefore advantageous to use as a therapeutic.

In vivo delivery of siRNAs faces several biological barriers that hinder their accumulation in the target tissue and cells. To promote accumulation of siRNA at the target site, siRNAs are loaded onto delivery vehicles such as nanoparticles. Based on their chemical composition, nanoparticles can be categorized into lipid based, polymeric or inorganic, each with their own advantages and limitations. Nanoparticles can be synthesized to a given size and functionalized with additional chemical moieties. The typical 20 – 200 nm size allows for avoidance of renal

clearance and extravasation through the enhanced permeability and retention effect. The addition of a targeting moiety such as ligand or antibody favors their binding and uptake in target cells. Polymeric nanoparticles with amines promote endosomal release through the proposed proton-sponge effect. Despite current advances in nanoparticle development, improving siRNA release and accumulation in target cells remain a challenge and an area of intense research. Thus, our lab has developed a hybrid nanoparticle platform that takes advantages of inorganic nanoparticle core, cationic polymer, PEG layer, as well as antibody targeting agent for effective delivery of HER2 targeting siRNA to treat HER2+ breast cancer (239).

In chapter 2, I described the selection of the most effective HER2 targeting siRNA sequence and the *in vitro* and *in vivo* characterization. Selection criteria include silencing and anti-proliferative efficacy in HER2+ cancer cell lines, the number of potential off-targets, and effect on HER2- and normal cell lines. The final sequence, designated siHER2, had the highest gene silencing and growth inhibition effect among 76 potential candidates in a panel of five HER2+ cancer cell lines but the least number of potential off-targets when searched through BLASTN. Using siHER2, I showed significant knockdown of HER2 protein via immunofluorescence microscopy and western blot, which also induced cell cycle arrest at the G1 phase and apoptosis.

To demonstrate the ability of siHER2 to overcome intrinsic resistance to trastuzumab and lapatinib, I compared side-by-side cellular response to siHER2,

trastuzumab and lapatinib in three HER2+ cancer cell lines with varying degree of sensitivity to trastuzumab and lapatinib. In JIMT1 cells, which are resistant to both trastuzumab and lapatinib, significant growth inhibition was achieved using 10 nM of siHER2. These experiments also revealed that response to HER2 inhibition is inversely correlated with HER2 expression. To validate this finding, I measured the 50% growth inhibition value of siHER2, lapatinib and trastuzumab in 13 additional HER2+ cell lines and found the same pattern. Therefore, cells with higher HER2 expression are generally more sensitive to HER2 silencing and inhibition by trastuzumab and lapatinib. These results agree with the notion that cells expressing high level of HER2 are likely more dependent on HER2 signaling for proliferation and survival. However, in all cases, 10 nM of siHER2 was able to inhibit the growth of all HER2+ cancer cell lines tested, suggesting that siHER2 is a far more potent therapeutic than trastuzumab or lapatinib. We also conclude that despite some cell lines having lower HER2 expression than others do and are likely less dependent on this oncogene, they nonetheless cannot survive when HER2 expression is knocked down to below a certain threshold using siRNA.

To mimic acquired resistance observed in the clinics, I grew the HER2+ cell line BT474 in the presence of trastuzumab or lapatinib for over 6 months. Resistance populations were generated and I again compared the cells' response to siHER2, trastuzumab and lapatinib. Similar to the intrinsically resistant cells, the acquired resistant BT474 populations remain sensitive to siHER2. Western blot of the downstream signaling pathway revealed that siHER2 could block the phosphorylation of AKT and ERK, while trastuzumab or lapatinib could not. These

results suggest that acquired resistant cells remain dependent on HER2 and that the high potency of siHER2 allowed for a more complete inhibition of HER2 signaling.

Δ16HER2 results from the alternative splicing of HER2 mRNA where exon 16 is skipped, resulting in a form that readily homodimerizes and is more oncogenic than the wild type. To show that siHER2 can also target this variant, I used real-time PCR and primer specific for Δ16HER2 to validate the silencing efficacy. Both the wild type and Δ16HER2 were knocked down by over 75%.

Finally, to show that siHER2 has efficacy *in vivo*, I compared the anti-tumor effect of trastuzumab and siHER2 in a mouse xenograft model based on a version of trastuzumab resistant BT474 cells. SiHER2 was complexed to mesoporous silica nanoparticles developed in our group. These particles have a core size of 50 nm and are further functionalized with a layer of the cationic polymer polyethylenimine for siRNA binding and endosomal release. A layer of polyethylene glycol is then added to reduce toxicity associated with polyethylenimine and prevent phagocytosis from macrophages. Finally, trastuzumab was added to the surface as a targeting agent and the final particle size in water is approximately 100 nm. A saline and non-targeting control siRNA on nanoparticles groups were included as control. All treatments were administered via intravenous injection. Only the siHER2 group showed significant tumor growth inhibition over a span of 45 days. Therefore, siHER2, when delivered by nanoparticles, has promising *in vivo* activity that can overcome trastuzumab resistance and warrants further investigation.

Chapter 3 reports the finding that long-term siHER2 treatment does not lead to significant resistance. In this study, I repeatedly transfected BT474 cells with siHER2 using the commercial transfection reagent Dharmafect and nanoparticles with or without trastuzumab. A fraction of cells was saved at the end of 8th, 15th and 30th week for characterization. Endpoint cell viability assays showed that the treated populations still respond to siHER2 with minimal loss of sensitivity. Only the group treated with siHER2 on nanoparticles with trastuzumab showed a more pronounced change, which was limited to 10%. These cells also remained sensitive to trastuzumab and lapatinib. In contrast, resistant BT474 cells generated from the previous study had lost several order of magnitude in their sensitivity to trastuzumab or lapatinib. Therefore, within a period of approximately 6 months, siHER2 did not induce resistance the way trastuzumab or lapatinib did, and therefore has a more durable therapeutic effect.

Further phenotypic characterization of the siHER2 treated cells showed that they did not undergo epithelial to mesenchymal transition, a process that is associated with drug resistance and metastasis. Surface CD24 and CD44 antigens staining also revealed no enrichment in the tumor initiating cell population. Reverse phase protein microarray analysis of protein samples showed that the lapatinib resistant BT474 cells had high HER2 signaling in the presence of the inhibitor. Expression of ER and phosphorylation of focal adhesion kinase was also upregulated. ER signaling was shown in other studies to confer resistance to lapatinib. Focal adhesion kinase was also implicated in promoting receptor cross

talk by mediating clustering of HER2 with other receptors. These changes were not observed in the long-term siHER2 treated cells, where HER2 signaling also remained suppressed in the presence of siHER2.

Conclusions and Future Directions

In conclusion, I demonstrated that siHER2 is more effective than trastuzumab and lapatinib. Not only can it overcome resistance to trastuzumab and lapatinib, long-term exposure to siHER2 does not readily induce acquired resistance. Knocking down genes at the mRNA level blocks the synthesis of HER2 protein altogether, which provides a more complete inhibition than inhibitors or antibodies do.

Other than surface receptors such as HER2, siRNAs can also target intracellular proteins and those for which inhibitors and antibodies cannot be readily developed, as in the case of the RAS and MYC oncogenes. The high specificity of siRNAs would result in greater efficacy and lower toxicity compared to small molecule inhibitors.

The MSNP delivery platform developed in our group has the potential to carry different siRNAs on the same particle. It is therefore possible to co-target multiple surface receptors and kinases in the downstream pathway, thereby exerting a greater inhibitory effect and preventing resistance associated with alternative signaling pathways and decoupled downstream signaling. For example, the HER family receptors, which include EGFR, HER3 and HER4, may be co-targeted with a cocktail of siRNAs to prevent the activation of non-HER2 dimers.

In addition, the co-targeting of PI3K and AKT can block their hyper-activation associated with mutation and feedback loop.

In addition to siRNAs, our MSNPs can also hold chemotherapeutic drugs within the silica core. Therefore, future investigation can study the co-delivery of chemotherapeutic compounds and siRNAs to maximize their therapeutic potential. One example is the co-delivery of siHER2 and docetaxel, which mirrors the first line treatment for metastatic HER2+ breast cancer consisting of two HER2 antibodies (trastuzumab and pertuzumab) plus a taxane. This combination requires over 6 hours of infusion and costs over $250,000 per treatment (drug cost alone). Delivering both siHER2 and docetaxel on the same nanoparticle can reduce the infusion time, hence cutting significant hospital cost. This treatment would be more patient-friendly as well. Another example is co-delivering a chemotherapy and an siRNA against the efflux pump proteins such as the multi-drug resistance protein (MDR1). The MDR1 protein reduces the efficacy of chemotherapeutics by actively pumping out the drugs. Interestingly, it has been shown that simply delivering chemotherapeutic drugs using nanoparticles could already partially bypass the effect of efflux pumps due to their distinct cellular uptake mechanism and subsequent cytosolic localization (284). One may also consider knocking down anti-apoptotic proteins such as BCL2, BCL-XL, and Survivin to enhance the cytotoxic effect of chemotherapeutics that are delivered on the same nanoparticles.

Lastly, we have reported that protonated MSNPs have antioxidant properties and could limit the metastatic potential (migration and invasion) of triple

negative breast cancer cells MDA-MB-231(283). It would be interesting to explore how they affect HER2+ breast cancer cells and whether they can prevent metastasis *in vivo*. All these topics can serve as extensions of our existing studies toward translation of our versatile nanoparticle delivery platform to various clinical applications.

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