이학박사학위논문

HER2 과발현 암 환자 치료를 위한 동반 상승효과 항체 병용에 대한 연구

Studies of Synergistic Antibody Combination for Treatment of Patients with HER2-Positive Cancer

2015년 2월

서울대학교 대학원

생명과학부

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지도교수 **박 동 은**

이 논문을 이학박사 학위논문으로 제출함

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고 봉 국

고봉국의 박사학위논문을 인준함

2014년 12월

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Studies of Synergistic Antibody Combination for Treatment of Patients with HER2-Positive Cancer

A dissertation submitted in partial fulfillment
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To the Faculty of
School of Biological Sciences
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by

Bong-Kook Ko

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ABSTRACT

Studies of Synergistic Antibody Combination for Treatment of Patients with HER2-Positive Cancer

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Biological Sciences

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Seoul National University

Gastric cancer is one of the leading types of cancer worldwide. Although the trend in death rates for gastric cancer is decreasing, prognosis is poor and few therapeutic options are available, particularly in advanced stages. The frequency of HER2-positive gastric cancer is 22.1% based on ToGA trial. Although reports are conflicting, some studies have suggested that HER2-positive status in gastric cancer is associated with poor outcomes and aggressive disease. Trastuzumab, trade name HERCEPTIN, is a HER2-targeting therapeutic antibody and approved for the treatment of HER2-overexpressing breast cancer. Currently, trastuzumab is also approved for the treatment of HER2-overexpressing metastatic gastric

cancer based on ToGA trial that proved the clinical benefit of combination treatment of trastuzumab and chemotherapy. Median overall survival was 13.8 months in trastuzumab plus chemotherapy compared with 11.1 months in those assigned to chemotherapy alone. Nonetheless, improving therapeutic efficacy and patient survival is important, particularly in patients with HER2-positive gastric cancer. Recent evidence suggests that particular combinations of non competing antibodies targeting the same receptor increase antitumor activity *in vitro* and *in vivo*. One example is the combination of pertuzumab, which binds to sub-domain II of the extracellular domain (ECD) of HER2, with trastuzumab, which binds to sub-domain IV. Pertuzumab, which has limited antitumor activity as a single agent in HER2-overexpressing breast cancer cells, shows increased efficacy in combination with trastuzumab. The increased efficacy of antibody combinations has also been demonstrated with EGFR-targeting or VEGFR3-targeting antibodies.

In this thesis, I have investigated on the efficacy and mechanism of a novel HER2-targeting monoclonal antibody 1E11 in HER2-positive gastric and breast cancer. 1E11 shows significant antitumor activity as a single agent in *in vitro* and *in vivo* HER2-positive gastric cancer models. Antitumor activity of 1E11 is increased in a highly synergistic manner in combination with trastuzumab. The two antibodies bind to sub-domain IV of the receptor, but have non-overlapping epitopes, allowing them to simultaneously bind to HER2. Treatment with 1E11 alone induced apoptosis in HER2-positive cancer cells, and this effect was enhanced by combination treatment with trastuzumab. Combination treatment with 1E11 and trastuzumab reduced the levels of total HER2 protein and those of aberrant HER2 signaling molecules including phosphorylated HER3 and EGFR. Since

1E11 is murine-derived monoclonal antibodies, murine variable region could be immunogenic in human. So I performed humanization by CDR-grafting of murine CDRs of 1E11 to human antibody germline framework. The humanized antibody by this approach, hz1E11, shows almost identical affinity and biological activity to the parental antibody. To improve the affinity of hz1E11, I performed affinity maturation by site-directed mutagenesis in CDR3 of heavy and light chain. Using antibody phage display technology, I selected 1A12 clone which has 4 amino acid mutations in CDR-L4 and shows improved affinity more than 10-fold. I confirmed that antitumor activity of 1A12 is correlated with HER2 levels in more than 15 gastric and breast cancer cell panels. The antibody-dependent cellular cytotoxicity (ADCC) activity of 1A12 is comparable to that of trastuzumab and pertuzumab. In combination setting with trastuzumab, 1E11 completely inhibits tumor growth in OE-19 xenograft model, while pertuzumab causes partial inhibition of tumor growth.

In conclusion, 1E11 showed synergistic antitumor activity in combination with trastuzumab in HER2-positive human gastric cancer *in vitro* and *in vivo*. The antitumor activity of 1E11 was mediated by its apoptotic activity and the inhibition of HER2 homo-and hetero-dimerization downstream signaling. This antibody combination could be a novel potent therapeutic strategy for the treatment of patients with HER2-overexpressing gastric and breast cancer.

Keywords: HER2, Gastric cancer, Antibody, Combination, Synergism

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

BACKGROUND

1. Epidermal Growth Factor Receptor 2 (HER2) in Gastric Cancer

Gastric cancer is one of the leading types of cancer worldwide. Although the trend in death rates for gastric cancer is decreasing (Siegel et al., 2012), prognosis is poor and few therapeutic options are available, particularly in advanced stages. Since most of symptoms for this type of cancer are nonspecific and screening strategies are absent in some countries, most patients with gastric cancer are developed locally advanced or metastatic disease during their illness. The treatment for advanced gastric cancer is mainly palliative and based on systemic chemotherapy, with median overall survival (OS) rarely exceeding 12 months and 5-year survival rate is less than 7% (Gomez-Martin et al., 2014 and Pasini et al., 2011). The predominant histological type of gastric cancer is adenocarcinoma, 95% of tumors, and the main sub-types are intestinal, diffused and mixed type.

Radical gastrectomy is the only curative treatment of gastric cancer, but recurrences are commonly observed in approximately 60% of patients (Buzzoni et al., 2006). For advanced stage patients, systematic chemotherapy is the mainstay of treatment (Field et al., 2008). Although recent studies showed some benefits from chemotherapy regiments including docetaxel, capecitabine, irinotecan,

cisplatin and oxaliplatin, there is no internationally accepted standard of care (Bilici, 2014). To date, molecular targets such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), vascular endothelial growth factor receptor 2 (VEGFR2), and hepatocyte growth factor receptor (HGFR/c-Met) have been tested by clinical trials in metastatic gastric cancer (Ku et al., 2010). A recent phase III trial proved the benefit of HER2-targeting antibody (Bang et al., 2010) and VEGFR2-targeting antibody (Wilke et al., 2014). Despite these remarked advances with targeted therapy, the prognosis of patients with advanced gastric cancer remains poor. Therefore, new therapeutic options are required to improve the survival of patients (Wagner and Moehler, 2009).

Overexpression/amplification of HER2 in breast cancer, HER2-positive subtypes, and its relationship with very poor prognosis are well established in breast cancer (Slamon et al., 1987 and Varley et al., 1987). HER2-positive breast cancer is also associated with increased risk of local growth and distinct metastasis. There are many studies indicating that HER2 is also present in other cancers, particularly in gastric cancer (Semba et al., 1985 and Tsuda et al., 1989).

The frequency of HER2-positive gastric cancer is 22.1% based on ToGA trial (Bang et al., 2010). Since the examination method and objective criteria vary between studies, the frequency of HER2-positive gastric cancer varies in ranging from 6.0 to 29.5% in early studies. The investigators in ToGA trial conducted a validation

study to assess the immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) protocols for testing HER2 status in advanced gastric cancer (Hofmann et al., 2008). Tissue specimens from 3,807 patients, in 24 countries, were collected and analyzed at a central laboratory using both IHC and FISH methods and HER2-positive gastric cancer is defined as IHC 3+ or FISH-positive (Bang et al., 2010). There were no marked racial differences in HER2 expression; instead, differences in HER2 expression were mainly attributed to the site of primary tumor, gastric vs. gastroesophageal junction, and histological type. Since the IHC staining pattern of gastric cancer is different from breast, the criteria for HER2 status also differ between breast cancer and gastric cancer (Hofmann et al., 2008). HER2-positive gastric cancer is defined as IHC 3+ or FISH-positive in the USA and Japan, while it is defined as IHC 3+ or as IHC 2+ plus FISH-positive in Europe (Albarello et al., 2011). There should be wider discussion about the algorithm and criteria to define the HER2-positivity in gastric cancer.

Initial studies suggested a negative effect of HER2 expression level on overall survival in gastric cancer (Okines et al., 2010 and Yonemura et al., 1991). However, the studies in gastric cancer to date have yielded inconsistent relationship between HER2 overexpression and bad prognosis (Boku, 2014). Some studies showed that HER2 positivity was associated with a significantly worse prognosis (Kim et al., 2012; Park et al., 2006; Tanner et al., 2005), whereas others found no association between HER2 status and prognosis

(Gomez-Martin et al., 2012 and Terashima et al., 2011). In some case, median overall survival was longer in HER2-positive than in HER2-negative patients (Janjigian et al., 2012 and Gomez-Martin et al., 2012). Therefore, the relationship between HER2 status and prognosis of gastric cancer patients remains controversial.

Figure 1. Mechanism of homo- and hetero-dimerization of the extracellular domain of ErbB receptors.

The unliganded extracellular region of EGFR exists as tethered and extened, but inactive status. Ligand binding stabilizes the extended conformation and induces homo- and/or heterodimerization. HER3 dimerization also occurs very similarly to that of EGFR. However, HER3 could form only heterodimer because extracellular region of HER3 does not homodimerize in solution or in cells. Extracellular region of HER2 exists as stable extended form, so could dimerize with other HER2 or ligand-induced EGFR or HER3.

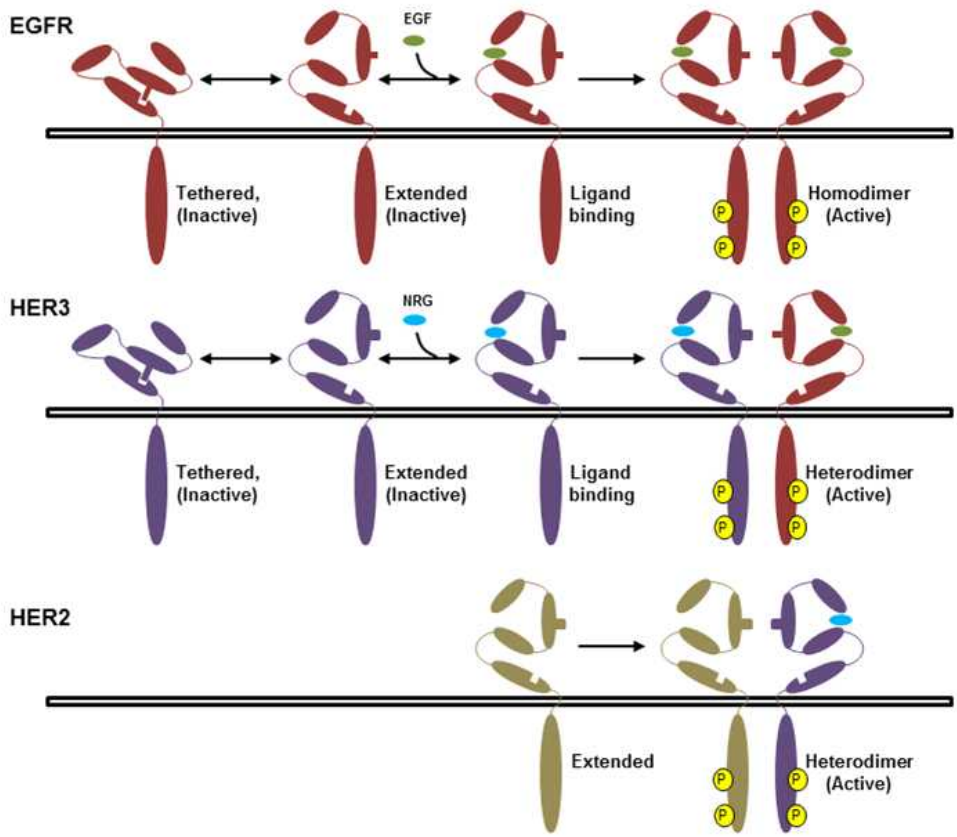
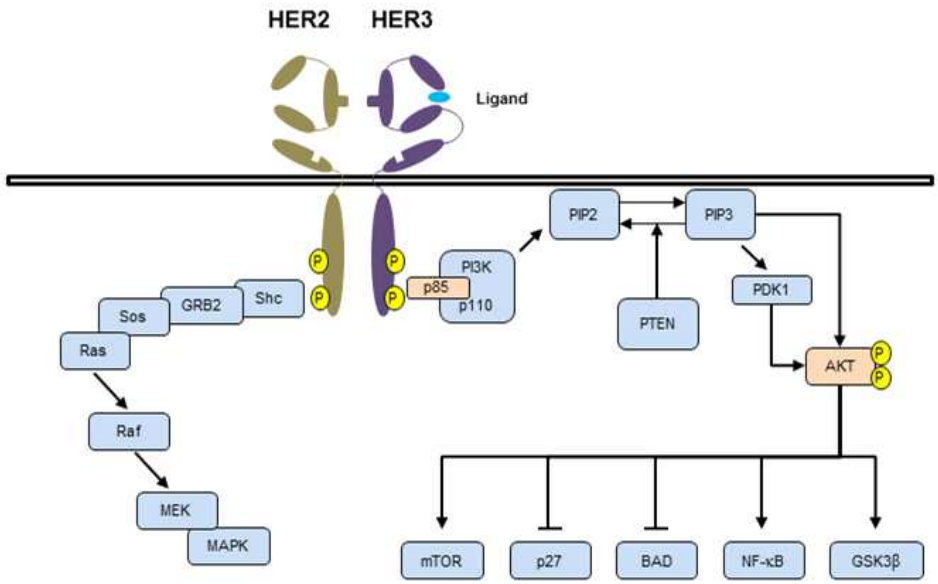

EGFR
EGF
Tethered,
(Inactive)
Extended
(Inactive)
Ligand
binding
Homodimer
(Active)
HER3
NRG
Tethered,
(Inactive)
Extended
(Inactive)
Ligand
binding
Heterodimer
(Active)
HER2
Extended
Heterodimer
(Active)

Figure 2. Downstream signaling of HER2/HER3 dimerization

HER2/HER3 dimerization by ligand, e.g., neuregulin, initiates signaling through Ras/Raf/MEK/ERK pathway, which promotes tumor cell survival, and the PI3K/AKT/mTOR pathway, which stimulates proliferation. Activation of MAPK pathway leads to the transcription of genes that drive cellular proliferation as well as migration, differentiation and angiogenesis. Signaling through the PI3K/AKT pathway leads to several cellular end points, and survival and anti-apoptosis signaling are two main outcomes.


HER2
HER3
Ligand
P
P
P
P
Shc
GRB2
Sos
Ras
Raf
MEK
MAPK
PI3K
p85
p110
PIP2
PIP3
PTEN
PDK1
AKT
P
P
mTOR
p27
BAD
NF-κB
GSK3β

2. HER2-Targeted Antibody Therapy

HER2 is a type 1 transmembrane receptor tyrosine kinase (RTK) and a member of the epidermal growth factor receptor (EGFR/ErbB) family, which includes four receptors: EGFR (HER1), c-erbB2 (HER2), c-erbB3 (HER3), and c-erbB4 (HER4). Members of EGFR family comprise of three domains: extracellular domain corresponding to the ligand binding site, α-helical transmembrane domain, and intracellular domain having a tyrosine kinase activity and phosphorylation sites (Olayioye et al., 2000). ErbB family proteins, exception of HER2, normally folded and prevent the exposure of extracellular sub-domain II which is key region for receptor homo- and heterodimerization with other ErbB family receptors (Cho et al., 2002 and Burgess et al., 2003).

The extracellular domain initiates a conformational rearrangement by ligand binding, leading to the sub-domain II exposure (Fig. 1). The binding of ligand seems to stabilize these receptors in a conformation that allows dimerization (Ogiso et al., 2002). After receptor dimerization, cross-phosphorylation of the dimer partners by tyrosine kinase domain occurrs and docking sites are created that allow the recruitment of downstream signaling components and the formation of signaling complex (Mendelsohn et al., 2003). Although all four ErbB receptors have the same essential domains, the functional activity of each domain varies (Bagela and Swain, 2009). EGFR and ERBB4 have active tyrosine kinase domains and known ligands, however

ERBB3 can bind to several ligands but lacks intrinsic tyrosine kinase activity. By contrast, ERBB2 possesses an active tyrosine kinase domain but ligand has not been identified and exists as active conformation (Burgess et al., 2003 and Hynes and Lane, 2005). In this active conformation, sub-domains I and III are involved in folding the protein to expose the dimerization site so the ligand-binding site is unavailable.

EGFR, ERBB2 and ERBB3 are all implicated in the development and progression of cancer, however, the role of ERBB4 in oncogenesis remains less well defined (Bagela and Swain, 2009). Among the various homodimer and heterodimer of four receptors, the ERBB2/ERBB3 heterodimer is considered the most potent oncogenic signaling complex with respect to strength of interaction, ligand-induced tyrosine phosphorylation and downstream signaling (Holbro et al., 2003 and Pinkas-Kramarski et al., 1996). ERBB3 could form heterodimers but not homodimers (Berger et al., 2004).

HER2 induces the transduction of signaling *via* two major pathways, which are the Ras/Raf/MEK/ERK and the PI3K/AKT/mTOR pathways (Fig. 2). These pathways promote cell proliferation, inhibition of apoptosis, angiogenesis, and invasion, which lead to tumor growth and progression. Activation of these signaling pathway depends on the type of homodimers or heterodimers, and current study suggests that HER2 homodimers could only activate Ras/MAPK pathway, while heterodimers could activate both MAPK

and PI3K signaling pathways (Ghosh et al., 2011). Suppression of HER2-overexpression breast cancer by PI3K blockade proves the importance of PI3K-mTOR signaling (Yao et al., 2009). PI3Ks are heterodimers of a p85 regulatory subunit and p110 catalytic subunit. Among the three human p110 isoforms (alpha, beta, and delta), the p110 alpha catalytic subunit was recently shown to be required for HER2-dependent mammary tumorigenesis in mice (Utermark et al., 2012). In addition to PI3K signaling, MAPK signaling appears to contribute to progression of HER2-positive breast cancer. Overexpression of HER2 in estrogen receptor (ER) positive MCF-7 cells caused hyperactivation of MAPK signaling and resistance to the ER modulator tamoxifen. Combination treatment of HER2 inhibitor and MEK inhibitor increased tamoxifen sensitivity and suppressed MAPK signaling in association with reduced MCF7/HER2 xenograft tumor growth (Kurokawa et al., 2000). Since combined administration of PI3K/mTOR inhibitor and MEK inhibitor achieved nearly complete tumor regression in xenograft model, strategies that combine inhibition of PI3K and MEK may achieve the most benefit against HER2-overexpressing cancers, particularly those refractory to HER2-targeted treatments (Roberts, et al., 2012).

Antibody-based therapy for cancer has become established over the past 15 years and is now one of the most successful and important strategies for treatment of patients with haematological malignancies and solid tumors (Scott et al., 2012). The mechanism of tumor cell killing by antibody can be summarized as direct action,

immune-mediated action, and specific effects (Fig. 3). Antibodies directly show antitumor activity through receptor blockade or agonistic activity, induction of apoptosis, or delivery of a drug or cytotoxic agent as antibody-drug conjugate (ADC). The immune-mediated cell killing mechanisms of antibody include complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and regulation of T cell function. Some antibodies show antitumor activity through tumor vascular and stromal cell ablation by vasculature receptor antagonism or ligand trapping. The Fc function of antibodies is particularly important for mediating tumor cell killing through ADCC and CDC.

Trastuzumab, trade name HERCEPTIN, is HER2-targeting therapeutic antibody and approved for the treatment of HER2-overexpressing breast cancer (Rosen et al., 2010). It is a standard-of-care treatment for metastatic breast cancer patients with HER2-overexpressing tumors, both as first-line treatment in combination with chemotherapy and as a single agent in women who have HER2-overexpressing metastatic breast cancer (MBC) that has progressed after chemotherapy for metastatic disease (Cobleigh et al., 1999 and Slamon et al., 2001). Currently, trastuzumab has also been approved for the treatment of HER2-overexpressing metastatic gastric cancer based on ToGA trial that proved the clinical benefit of combination treatment of trastuzumab and chemotherapy (Bang et al., 2010). In ToGA trial, 594 patients were randomly assigned to trastuzumab plus chemotherapy group (n = 298) and chemotherapy alone group (n =

296). Median overall survival was 13.8 months in trastuzumab plus chemotherapy compared with 11.1 months in those assigned to chemotherapy alone. The median progression-free survival of trastuzumab plus chemotherapy group is 6.7 months compared 5.5 months in control group. Overall tumor response rate (47% vs. 35%), time to progression (7.1 months vs. 5.6 months), and duration of response (6.9 months vs. 4.8 months) were significantly improved in the trastuzumab plus chemotherapy group.

The mechanisms of underlying the antitumor activity of trastuzumab in HER2-positive breast cancer are not completely known, but several have been proposed. Trastuzumab binds to extracellular sub-domain IV of HER2 (Fig. 4), thereby inhibits ligand-independent dimerization of the receptor and the subsequent activation of downstream signaling (Cho et al., 2003 and Junttila et al., 2009). Trastuzumab selectively inhibit HER2-HER3 heterodimerization and downregulates total levels of HER2 on the cell surface, reducing PI3K and MAPK signaling (Cuello et al., 2001 and Gajria and Chandarlapaty, 2011). Trastuzumab has also been shown to block the HER2 protein shedding, thus, preventing formation of the constitutively active membrane bound p95HER2 (Molina et al., 2001). Trastuzumab also induces cell cycle arrest by upregulation of $p27^{kip1}$ and inhibition of cdk2 activity (Le et al., 2003). Finally, HER2-positive cells are lysed through antibody-dependent cellular cytotoxicity, ADCC (Arnould et al., 2006 and Clynes et al, 2000).

Recent evidence suggests that particular combinations of noncompetitive antibodies targeting the same receptor increase antitumor activity *in vitro* and *in vivo*. One example is the combination of pertuzumab, which binds to sub-domain II of the extracellular domain (ECD) of HER2, with trastuzumab, which binds to sub-domain IV (Cai et al., 2008). Pertuzumab, which has limited antitumor activity as a single agent in HER2-overexpressing breast cancer cells, shows increased efficacy in combination with trastuzumab (Nahta et al., 2004). The benefits of the pertuzumab and trastuzumab combination were further demonstrated in preclinical and clinical trials (Baselga et al., 2010 and Scheuer et al., 2009). Pertuzumab has been approved for the treatment of HER2-positive metastatic breast cancer in combination with trastuzumab (Baselga et al., 2012). Other HER2-targeting antibodies showing better efficacy in combination than as single agents have been reported and have shown consistent downregulation of HER2 levels and beneficial combination effects in mouse models (Ben-Kasus et al., 2009). The increased efficacy of antibody combinations has also been demonstrated with EGFR-targeting antibodies (Friedman et al., 2005 and Koefoed et al., 2011).

Figure 3. Mechanisms of antitumor activity of therapeutic antibodies (Scott et al., 2012)

A, Direct tumor cell killing can be elicited by receptor agonist activity leading to apoptosis (represented by the mitochondrion), receptor antagonist activity, such as blocking dimerization and/or kinase activation and its downstream signaling, leading to reduced proliferation and apoptosis. An antibody binding to an enzyme can lead to neutralization, signaling abrogation and cell death, and conjugated antibodies can be used to deliver a payload (such as a drug, toxin, small interfering RNA or radioisotope) to a tumor cell. **B**, Immune-mediated antitumor activity can be carried out by the induction of phagocytosis, complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), genetically modified T cells being targeted to the tumor by single-chain variable fragment (scFv), T cells activation by antibody-mediated cross-presentation of antigen to dendritic cells, and inhibition of T cell inhibitory receptors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA4). **C**, Vascular and stromal cell ablation can be induced by vasculature receptor antagonism or ligand trapping (not shown), stromal cell inhibition, delivery of a toxin to stromal cells, and delivery of a toxin to the vasculature. MAC, membrane attack complex; MHC, major histocompatibility complex; NK, natural killer.

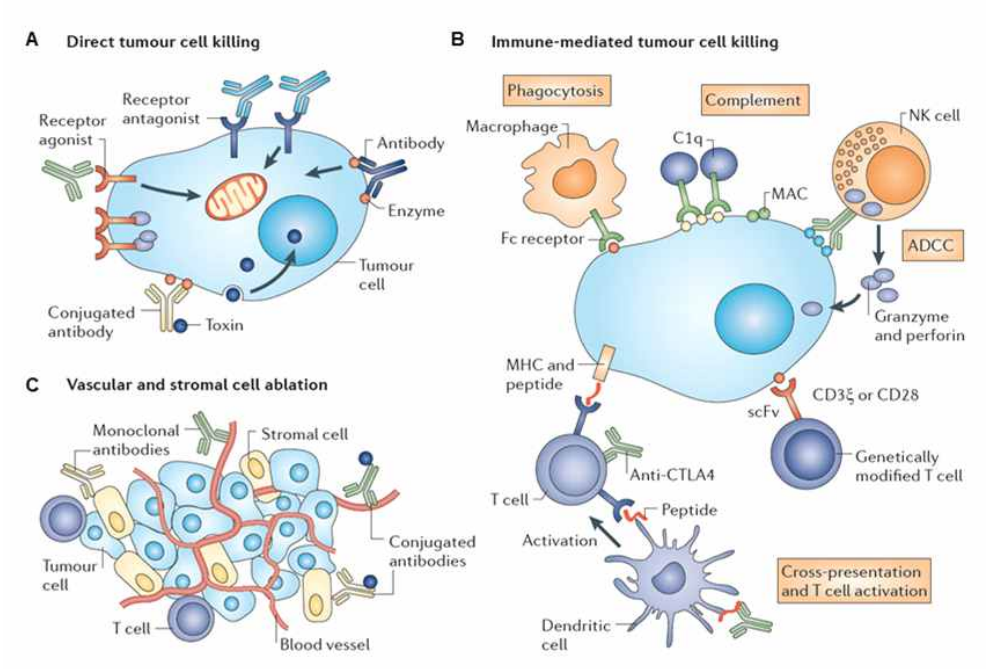
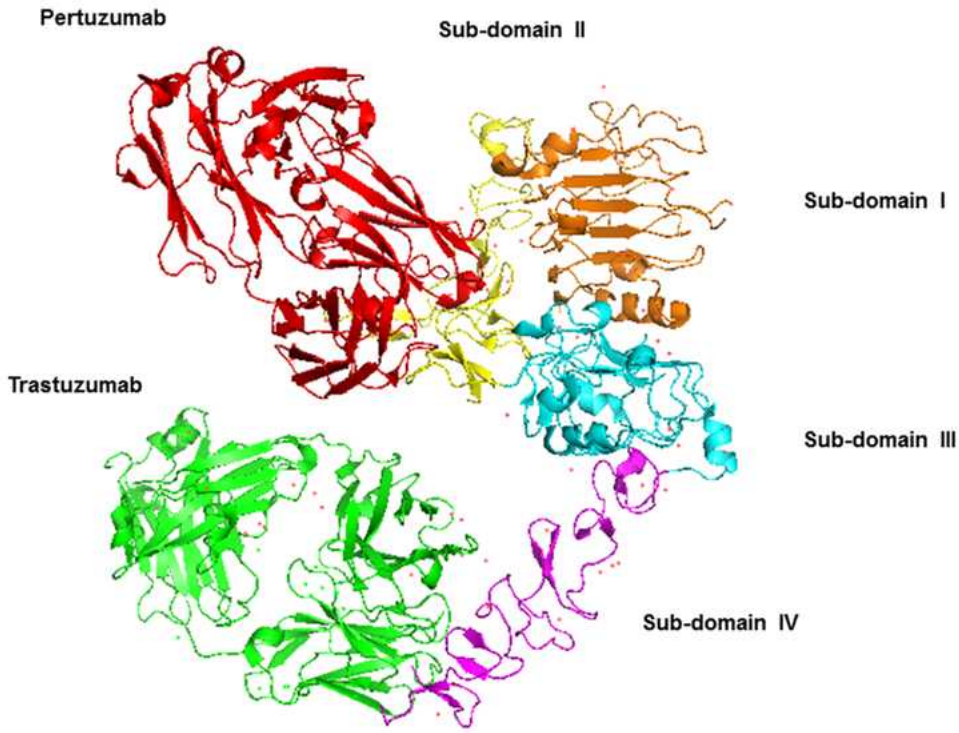

A
Direct tumour cell killing
Receptor antagonist
Receptor agonist
Antibody
Enzyme
Tumour cell
Conjugated antibody
Toxin
B
Immune-mediated tumour cell killing
Phagocytosis
Complement
NK cell
Macrophage
C1q
MAC
Fc receptor
ADCC
Granzyme and perforin
MHC and peptide
CD3ξ or CD28
scFv
Genetically modified T cell
Anti-CTLA4
T cell
Peptide
Activation
Cross-presentation and T cell activation
Dendritic cell
C
Vascular and stromal cell ablation
Monoclonal antibodies
Stromal cell
Conjugated antibodies
Tumour cell
T cell
Blood vessel

Figure 4. Epitope of trastuzumab and pertuzumab

Trastuzumab binds to sub-domain IV, whereas pertuzumab binds to sub-domain II. Trastuzumab inhibits HER2 shedding and HER2 activation through ligand-independent HER2/HER3 dimerization. Since pertuzumab binds to sub-domain II responsible to dimerization, it inhibits HER2 dimerization with other ErbB family receptors. The molecular mechanism of HER2/HER3 dimerization is not completely known. Putative crystal structure of HER2-ECD/trastuzumab/pertuzumab is reconstructed with HER2-ECD/trastuzumab (PDB ID:1N8Z) and HER2-ECD/pertuzumab (PDB ID:1S78). Sub-domain of HER2-ECD is colored to brown, yellow, cyan, magentas for sub-domain I, II, III, and IV, respectively. Fab protein of trastuzumab and pertuzumab is colored to green and red, respectively.


Pertuzumab
Sub-domain II
Sub-domain I
Trastuzumab
Sub-domain III
Sub-domain IV

CHAPTER I

Combination of novel HER2 targeting antibody 1E11 with trastuzumab shows synergistic antitumor activity in HER2-positive gastric cancer

Abstract

The synergistic interaction of two antibodies targeting the same protein could be developed as an effective anti-cancer therapy. Human epidermal growth factor receptor 2 (HER2) is overexpressed in 20 - 25% of breast and gastric cancer patients, and HER2-targeted antibody therapy using trastuzumab is effective in many of these patients. Nonetheless, improving therapeutic efficacy and patient survival are important, particularly in patients with HER2-positive gastric cancer. Here, I describe the development of 1E11, a HER2-targeted humanized monoclonal antibody showing increased efficacy in a highly synergistic manner in combination with trastuzumab in the HER2-overexpressing gastric cancer cell lines NCI-N87 and OE-19. The two antibodies bind to sub-domain IV of the receptor, but have non-overlapping epitopes, allowing them to simultaneously bind HER2. Treatment with 1E11 alone induced apoptosis in HER2-positive cancer cells, and this effect was enhanced by combination treatment with trastuzumab. Combination treatment with 1E11 and trastuzumab reduced the levels of total HER2 proteins and those of aberrant HER2 signaling molecules including phosphorylated HER3 and EGFR. The synergistic antitumor activity of 1E11 in combination with trastuzumab indicates that it could be a novel potent therapeutic antibody for the treatment of HER2-overexpressing gastric cancers.

Introduction

Gastric cancer is one of the most common cancers, with approximately one million new cases diagnosed each year; it is the third leading cause of cancer death in both sexes worldwide (Ferlay et al., 2012). Despite advances in the prevention and treatment of gastric cancer and a decrease in mortality rates, the prognosis of patients with gastric cancer remains poor and few effective therapeutic options are available, in particular for advanced stages (Siegel et al., 2012). The 5 year survival rate in most parts of the world is approximately 20%. HER2 is a receptor tyrosine kinase and a member of the ErbB family, which includes EGFR, HER3, and HER4. This receptor family functions in the regulation of many essential cellular functions such as cell proliferation, differentiation, and apoptosis through homo- or hetero-dimerization and the activation of signal transduction pathways (Yarden and Sliwkowski, 2001). ErbB family proteins are overexpressed in several malignancies. In gastric cancer, overexpression of EGFR, HER2, and HER3 is correlated with poor prognosis (Garcia et al., 2003 and Hayashi et al., 2008). The HER2-targeting monoclonal antibody trastuzumab was first characterized *in vitro* and *in vivo* in 1992 (Kasprzyk et al., 1992) and was approved for the treatment of HER2-overexpressing metastatic breast cancer in 1998. Recently, the ToGA trial (Trastuzumab for Gastric Cancer) demonstrated the survival benefit

of trastuzumab in HER2-overexpressing gastric cancer patients (Bang et al., 2010) after Food and Drug Administration approval of this antibody for the treatment of HER2-positive metastatic gastric and gastroesophageal junction cancer.

Recent evidence suggests that particular combinations of not competing antibodies targeting the same receptor increase antitumor activity *in vitro* and *in vivo*. One example is the combination of pertuzumab, which binds to sub-domain II of the extracellular domain (ECD) of HER2, with trastuzumab, which binds to sub-domain IV (Cai et al., 2008 and Cho et al., 2003). Pertuzumab, which has limited antitumor activity as a single agent in HER2-overexpressing breast cancer cells, shows increased efficacy in combination with trastuzumab (Nahta et al., 2004). The benefits of the pertuzumab and trastuzumab combination were further demonstrated in preclinical and clinical trials (Baselga et al., 2010 and Scheuer et al., 2009). Pertuzumab has been approved for the treatment of HER2-positive metastatic breast cancer in combination with trastuzumab (Baselga et al., 2012). Other HER2-targeting antibodies showing better efficacy in combination than as single agents have been reported to show consistent downregulation of HER2 levels and beneficial combination effects in mouse models (Ben-Kasus et al., 2009). The increased efficacy of antibody combinations has also been demonstrated with EGFR-targeting antibodies (Friedman et al., 2005 and Koefoed et al., 2011).

In the present study, I report the development of a novel HER2-targeted antibody termed 1E11 and describe its anti-cancer activities as a single agent and in combination with trastuzumab in preclinical models. 1E11 had moderate efficacy in HER2-overexpressing NCI-N87 and OE-19 gastric cancer cells; however, its efficacy increased dramatically *in vitro* and *in vivo* when used in combination with trastuzumab, showing a highly synergistic effect. The binding site of 1E11 was localized to sub-domain IV, at a distinct epitope different from that of trastuzumab. 1E11 induced apoptosis in combination with trastuzumab, which showed weak apoptotic activity as a single agent, and combination treatment with 1E11 and trastuzumab inhibited epidermal growth factor (EGF) and HRG1-induced cell proliferation. The results of the present study suggest that HER2-targeting antibody combinations are valid therapeutic strategies for the treatment of HER2-overexpressing gastric cancer, and 1E11 is a strong synergistic partner for trastuzumab-based combination treatments.

Materials and Methods

Cell lines and materials

BT-474, SK-BR-3, NCI-N87, KATO-III, Hs746T, HCC-202, HCC-1954, and MCF-7 cells were purchased from American Type Culture Collection (ATCC). OE-19 cells were obtained from the European Collection of Cell Culture (ECACC). MKN-7 cells were from the Japanese Collection of Research Bioresources (JCRB), and MKN-45, SNU-216, MDA-MB-231, and MDA-MB-453 cells were from the Korean Cell Line Bank (KCLB). JIMT-1 cells were gifted from Asan Medical Center in Seoul Korea. Cell culture media were Dulbecco's Modified Eagle's Medium (DMEM): F-12 for BT-474 cells, DMEM for Hs746T cells, and RPMI-1640 for all other cell lines. All media were supplemented with 10% fetal bovine serum (FBS), and antibiotics and cells were cultured at 37°C under 5% CO_2. Trastuzumab and pertuzumab were produced by Genentech Incorporated, and palivizumab was produced by MedImmune, LLC. ChromPure human IgG (Jackson Immunoresearch Lab) was used as human IgG control antibody in *in vitro* assays. 1E11 and oz1E11 antibodies were produced using the 293F system (Invitrogen) and purified using protein-A chromatography (GE Healthcare). The oz1E11 antibody (clone name: 1A12) consists of an optimized 1E11 antibody after humanization and affinity maturation. The endotoxin was removed with an Endotoxin removal kit (GenScript), and

endotoxin levels were determined using an Endotoxin detection kit (GenScript). Human HER3 and HER4 proteins were purchased from R&D systems and rhesus HER2, mouse HER2, and rat HER2 proteins were purchased from Sino Biological Inc.. Heregulin-1 (HRG1) and EGF proteins were purchased from R&D systems. Other recombinant proteins were produced as secretion proteins using the 293F system and purified using protein-A or Ni-NTA chromatography (Qiagen Inc.) for Fc-fused and His-fused proteins, respectively.

Cell viability assay

Cells were seeded in 96-well plates (Corning) in growth media containing 10% FBS and pre-cultured for 24 h. The cells were treated with antibodies at the indicated concentrations and culture for 3 - 6 days. For ligand-induced cell proliferation assays, NCI-N87 cells were seeded and pre-cultured for 24 h in 0.1% FBS media and treated with antibodies (10 mg/mL) for 1 h before the addition of ligand (200 ng/mL). Cell proliferation was assessed 3 days after the treatment. The WST (DoGen) or CellTiter-Glo (Promega) assay was used to measure cell viability. Relative cell viability was calculated by dividing the absorbance of each well by the mean absorbance of PBS-treated wells in each plate.

Surface Plasmon resonance (SPR) analysis

For epitope binning, trastuzumab and pertuzumab were immobilized onto separate CM5 sensor chip surfaces (GE Healthcare) using amine coupling at approximately 1000 response units (RU). HER2-ECD-His (320 nM) and antibodies (1 mg/mL) were sequentially coupled by binding for 4 min and stabilization for 5 min at 50 mL/min flow rate. For affinity measurements, goat anti-human IgG (γ) (Invitrogen) was immobilized onto a CM5 sensor chip using amine coupling, and antibodies were captured at approximately 50 RU. Then, HER2-ECD-His protein was injected at concentrations ranging from 0 to 640 nM. Sensorgrams were obtained at each concentration and evaluated using the BIAevaluation software.

Cell cycle and apoptosis analysis

NCI-N87 and BT-474 cells were treated with 10 mg/mL of antibodies in complete growth media. After 2 days of antibody treatment, cells were detached with trypsin and 1×10^5 cells were used for analysis. For cell cycle analysis, cells were fixed in 70% ethanol and resuspended in 33 mg/mL propidium iodide (PI) (Sigma-Aldrich Co.) supplemented with 1 mg/mL RNase A (Sigma-Aldrich Co.) and 10% Triton X-100. Samples were incubated for 30 min and analyzed by flow cytometry using a Cytomics FC500 (Beckman Coulter Inc). For apoptosis analysis, detached cells were stained with annexin V and PI

using the ApoScreen TM Annexin V Apoptosis kit (SouthernBiotech). Cell death was measured as cells staining positive for annexin V, PI, or both, as assessed by flow cytometry analysis.

Caspase-3/7 activity test

Cells were treated as described for the cell viability assay with 10 mg/mL of antibodies for 24 h. The Caspase-Glo 3/7 Assay (Promega) was used to measure caspase-3/7 activity. Caspase 3/7 activity was calculated as follows: Caspase 3/7 activity = (luminescent unit of treatment well - luminescent unit of blank well)/(mean luminescent unit of control well - luminescent unit of blank well).

Downstream signaling

Cells were treated as described in section 2.4 for 24 h, washed with ice-cold PBS, and lysed in a cell lysis solution [50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail (Sigma)]. Antibodies against HER2 (#4290), pHER2 (#2243), pHER3 (#4791), EGFR (#4267), pEGFR (#3777), AKT (#4691), pAKT (#4060), ERK (#4695), and pERK (#4370) were purchased from Cell Signaling Technology. Anti-HER3 (sc-285) antibody was purchased from SantaCruz Biotechnology and anti-GAPDH (AbC-1001) antibody was

purchased from AbClon. Horseradish peroxidase-conjugated anti-mouse (AbC-5001) and anti-rat (AbC-5003) antibodies were purchased from AbClon. Bands were visualized using AbSignal (AbClon, AbC-3001).

Antibody-dependent cellular cytotoxicity (ADCC) assay

SK-BR-3 cells were used as target cells, and human peripheral blood mononuclear cells (PBMCs) purified from the blood of five healthy donors were used as effector cells. Target cells (10,000) were incubated for 15 min with the indicated antibodies followed by the addition of effector cells at a ratio of 1:50 (target:effector) and incubation for an additional 20 h at 37°C under 5% CO_2. ADCC was determined using the EZ-LDH Cell Cytotoxicity Assay Kit (DoGen) according to the manufacturer's instructions. Cytotoxicity (%) was calculated using the following equation: (Experimental value - Effector Cell Spontaneous Control - Target Cell Spontaneous Control)/(Target Cell Maximum Control - Target Cell Spontaneous Control) × 100.

Optimization of 1E11 antibody

The humanized 1E11 antibody was developed by CDR-grafting into human germline genes. The homologous human germline genes with

highest sequence similarity were selected using IMGT/V-QUEST (Brochet et al., 2008). The IGKV1-39*01 and IGKJ1*01 genes for light chain and IGHV3-48*03 and IGHJ4*01 genes for heavy chain were selected as acceptor sequences for the grafting of murine CDRs. One residue in heavy chain H47 according to the Kabat numbering scheme was back-mutated to murine, since this site is responsible for stabilizing the CDR loop structure as well as modifying its position (Foote and Winter, 1992).

For affinity maturation of humanized 1E11, CDR3 residues in the heavy and/or light chain were randomized to 20 amino acids, and high affinity binders were selected using phage display. Selected clones were ranked based on the K_{off} value using SPR analysis with immobilized HER2-ECD protein. The oz1E11 antibody has four amino acid mutations in CDR3 of the parental light chain and showed a >10-fold improvement in K_{off} value.

NCI-N87 and OE-19 xenograft models

Athymic nude female mice (Daehan Biolink, Korea) were injected subcutaneously in the left flank area with 5×10^6 of NCI-N87 or OE-19 cells in Matrigel (BD Biosciences). Tumors were allowed to grow to approximately 200 mm^3 or 500 mm^3 in size, and mice were than randomized into groups. Animals received intraperitoneal administration of antibodies at the indicated doses twice weekly.

Tumor volumes were calculated using the formula (L×W×W)/2, where "L" represents the larger tumor diameter and "W" represents the smallest tumor diameter. Animals were sacrificed, and the tumors were isolated and weighted after the termination of studies. Animals in the study group were also sacrificed if the average tumor volume was >3,000 mm^3. Tumor xenograft tissues were resected and processed as formalin-fixed, paraffin-embedded specimen sections. The TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was performed to analyze apoptotic cell death. Cells were visualized under a light microscope.

All animal studies were conducted in accordance with the guidelines of the NIH "Guide for Care and Use of Animals" and an approved protocol received by the company's Institution Animal Care and Use Committee.

Statistical analysis

Statistical analysis was performed by Student unpaired t test to identify significant difference unless otherwise indicated. Differences were considered significant at a P value <0.05.

Results

1E11 synergistically inhibits gastric cancer cell growth in combination with trastuzumab

Mouse monoclonal antibodies against the ECD of HER2 were developed using our improved hybridoma technology. These clones were used to assess cell viability in a panel of gastric cancer cell lines including NCI-N87 and breast cancer cell lines including BT-474. 1E11 was selected based on its affinity for HER2 and efficacy against HER2-positive cancer cells, and was further developed into a mouse/human chimeric antibody with the IgG_1 kappa format.

1E11 and trastuzumab showed moderate antiproliferative activity against HER2-overexpressing gastric NCI-N87 cells as single agents, whereas in combination they showed dramatically increased antiproliferative activity (Fig. 5A). Pertuzumab did not show any substantial antiproliferative activity as a single agent or in combination with trastuzumab in NCI-N87 cells. The combination effect of 1E11 and trastuzumab was equivalent to that of trastuzumab and pertuzumab in HER2-overexpressing BT-474 breast cancer cells (Fig. 5B).

Combination effects were analyzed using the method of Chou and Talalay (Chou and Talalay, 1984) to obtain drug combination index

(C.I.) values. The combination effects of drugs are defined as synergism, addition, and antagonism according to C.I. values <1.0, equal to 1.0, or >1.0, respectively. The combination effect of 1E11 and trastuzumab was determined as strong synergism with C.I. values of 0.03, 0.05, and 0.08 at ED_{50}, ED_{75}, and ED_{90}, respectively.

The *in vitro* antiproliferative activity of 1E11 in combination with trastuzumab was confirmed *in vivo* in mice bearing NCI-N87 xenograft tumors. Both trastuzumab and 1E11 significantly reduced tumor volume and weight compared to control antibody when used as single agents (Fig. 6A and 6B); however, when used in combination, their antitumor activity was significantly higher than that of trastuzumab alone ($P < 0.0001$). The tumor growth inhibition (TGI) value of the antibody combination (95.1%) was much higher than those of trastuzumab and 1E11 as single agents (48.6% and 39.9%, respectively).

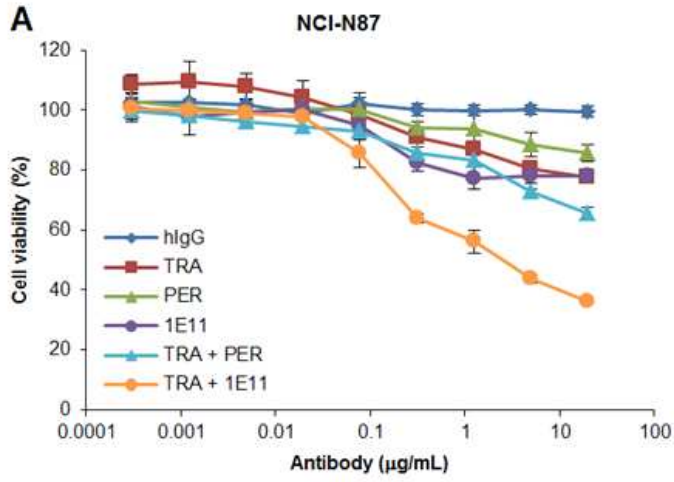
These results indicate that 1E11 in combination with trastuzumab inhibits HER2-overexpressing gastric cancer cell growth in a synergistic manner.

1E11 binds to domain IV of HER2 at a distinct epitope different from that of trastuzumab

To determine whether 1E11 binds to the same epitope as trastuzumab or pertuzumab, the binding of 1E11 to the HER2-ECD that was

Figure 5. 1E11 shows antiproliferative activity in combination with trastuzumab in a synergistic manner

A, NCI-N87 and **B**, BT-474 cells were treated with hIgG, trastuzumab (TRA), pertuzumab (PER), and 1E11 as single agents and in combination with trastuzumab in a 1:1 ratio for 4 days. Cell viability was expressed as the mean ± SD (n = 3), and the 100% point was defined as the absorbance of the untreated well.


A
NCI-N87
Cell viability (%)
120
100
80
60
40
20
0
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11
0.0001
0.001
0.01
0.1
1
10
100
Antibody (μg/mL)

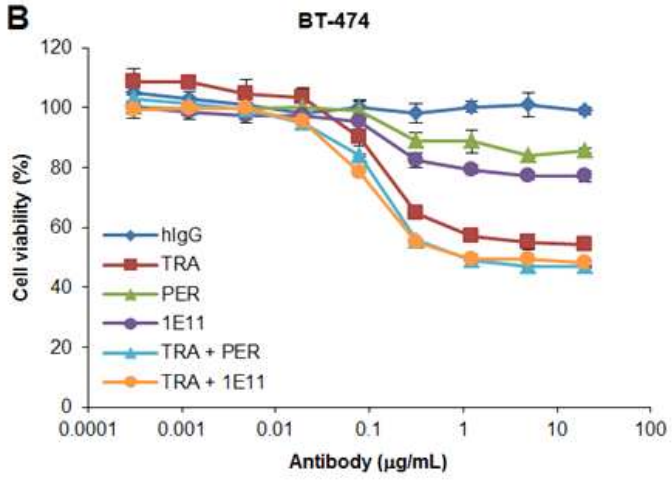
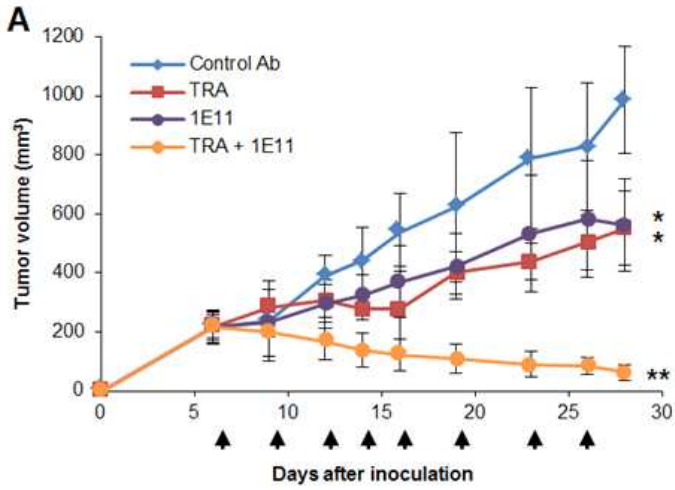
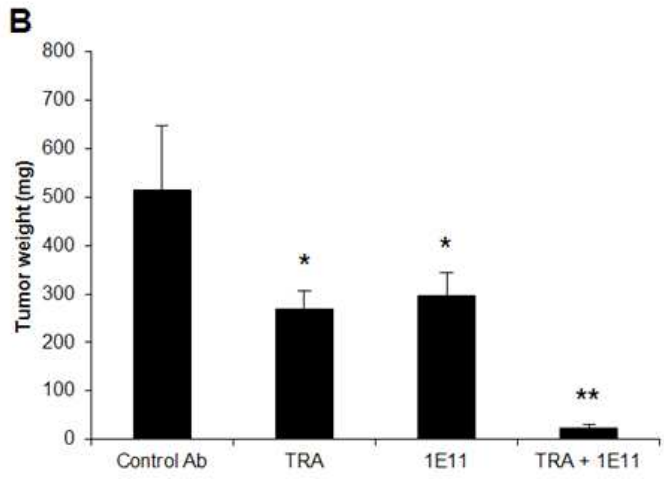

B
BT-474
Cell viability (%)
120
100
80
60
40
20
0
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11
0.0001
0.001
0.01
0.1
1
10
100
Antibody (μg/mL)

Figure 6. 1E11 shows antitumor activity in combination with trastuzumab in xenograft model

A, NCI-N87 cells were inoculated into mice (n = 5 mice/group each) and treatment with 10 mg/kg of antibody was started when tumor volumes reached approximately 200 mm^3. For combination treatment, 10 mg/kg of each antibody was administered. Palivizumab was used as the isotype control antibody. Administration days are indicated by arrows. Tumor volume (mm^3) was expressed as mean ± SD. **B**, Tumor masses were isolated after measuring tumor weight. Statistically significant differences were determined by Student's t test. *, $P < 0.01$ versus the control group and **, $P < 0.01$ versus the trastuzumab-treated group.


A
Control Ab
TRA
1E11
TRA + 1E11
Tumor volume (mm³)
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
Days after inoculation
*
*
**


B
Tumor weight (mg)
800
700
600
500
400
300
200
100
0
*
*
**
Control Ab
TRA
1E11
TRA + 1E11

occupied by trastuzumab or pertuzumab was analyzed by SPR. As shown in Figure 7A, 1E11 was able to bind a monomeric HER2-ECD-His protein captured by immobilized pertuzumab (Fig. 7A). These data indicate that 1E11 binds to a distinct epitope that is different from the epitopes of trastuzumab or pertuzumab, and that it is capable of simultaneous binding to HER2 with the two other antibodies.

Recombinant HER2-ECD domain fragments were used for epitope mapping of the three reagents (Fig. 7B), which showed that both 1E11 and trastuzumab bound to sub-domain IV. However, pertuzumab did not bind to domain II of recombinant HER2, suggesting that it was not able to mimic its natural conformation. To further characterize the 1E11 epitope, the binding of 1E11 to 15-mer peptides with a peptide-peptide overlap of 14 amino acids covering the whole HER2-ECD was examined. However, no significant binding signals were detected for any of the peptides (data not shown). Taken together, these results indicated that 1E11 binds to a discontinuous or conformational epitope in sub-domain IV.

Recombinant ErbB proteins were used to confirm that 1E11, similar to trastuzumab and pertuzumab, specifically binds HER2 and not the other human ErbB family members (Fig. 8A). The species cross-reactivity of 1E11 was determined by assessing the binding activity of 1E11 to HER2-ECD proteins from five different species. Similar to trastuzumab and pertuzumab, 1E11 bound to monkey HER2

Figure 7. 1E11 binds to a sub-domain IV epitope different from that of trastuzumab

A, Pertuzumab was immobilized on a CM5 sensor chip followed by sequential exposure to HER2-ECD-His, trastuzumab, and 1E11. **B**, The binding activities of antibodies were analyzed by ELISA using recombinant sub-domain proteins.

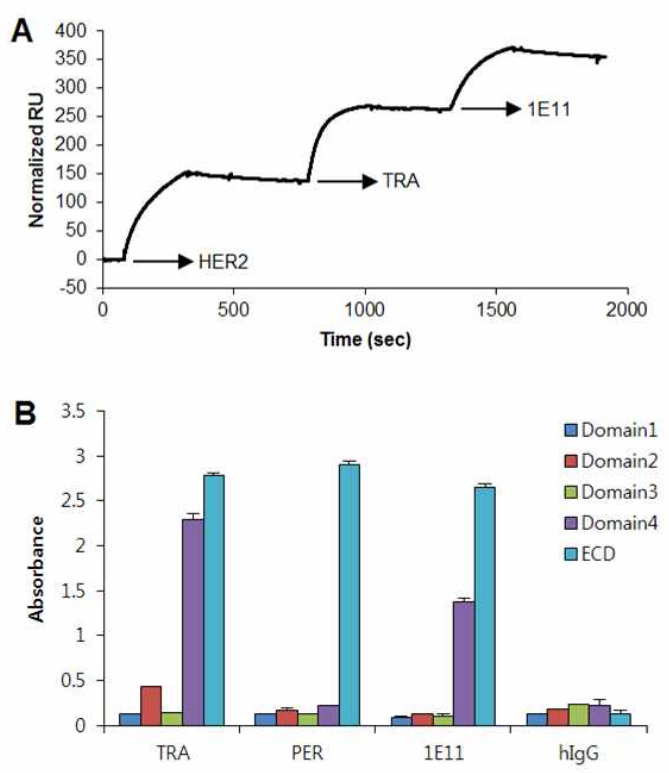
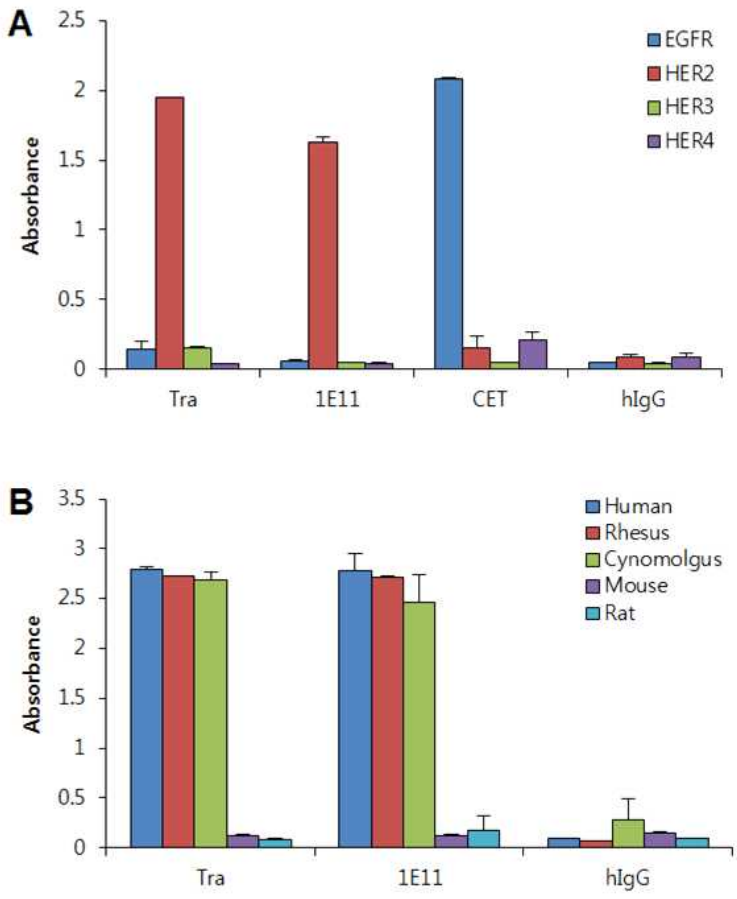

A
Normalized RU
400
350
300
250
200
150
100
50
0
-50
HER2
TRA
1E11
0
500
1000
1500
2000
Time (sec)
B
Absorbance
3.5
3
2.5
2
1.5
1
0.5
0
Domain1
Domain2
Domain3
Domain4
ECD
TRA
PER
1E11
hIgG

Figure 8. 1E11 specifically binds to HER2 in ErbB family and has species cross-reactivity with rhesus and cynomolgus monkey HER2 proteins

A, The ErbB family cross-reactivity of 1E11 was analyzed using recombinant human ErbB proteins. Cetuximab (CET) was used as the control antibody for the EGFR protein. **B**, The species cross-reactivity of 1E11 was analyzed using recombinant HER2 ECD proteins of the indicated species.


A
Absorbance
2.5
2
1.5
1
0.5
0
EGFR
HER2
HER3
HER4
Tra
1E11
CET
hIgG
B
Absorbance
3.5
3
2.5
2
1.5
1
0.5
0
Human
Rhesus
Cynomolgus
Mouse
Rat
Tra
1E11
hIgG

but not murine HER2 proteins (Fig. 8B).

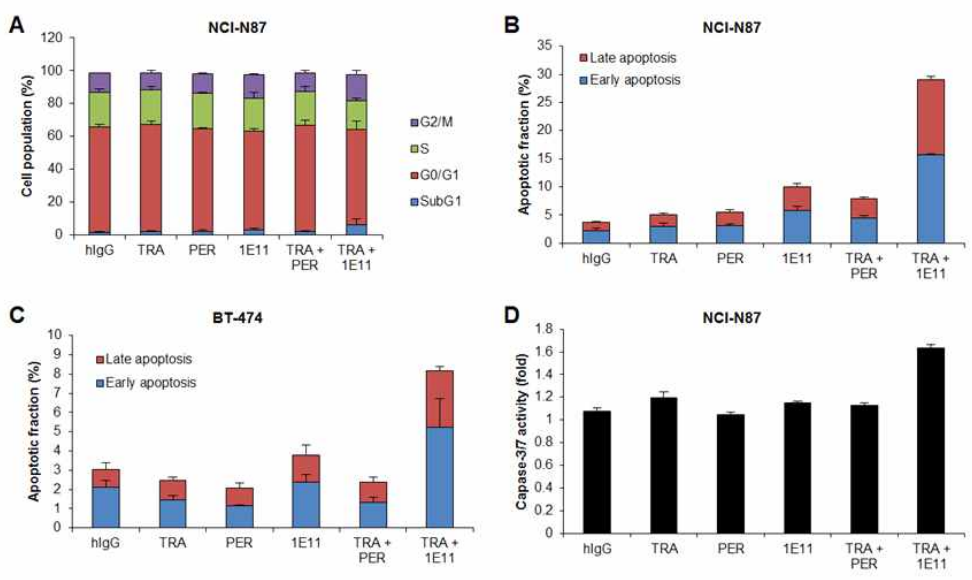
1E11 increases tumor cell apoptosis in combination with trastuzumab

To determine the mechanism underlying the antitumor activity of 1E11, cell cycle progression and apoptosis were analyzed by flow cytometry. Treatment of NCI-N87 cells with trastuzumab or 1E11 did not induce significant changes in the cell cycle either as single agents or in combination (Fig. 9A). However, combination treatment with 1E11 and trastuzumab increased the sub-G1 population of NCI-N87 cells, indicating cell death.

Assessment of cell apoptosis showed that 1E11 increased both the early and late apoptotic cell population compared to trastuzumab or pertuzumab single agent treatments, and this apoptotic activity was further increased in NCI-N87 cells exposed to combination treatment with 1E11 and trastuzumab (Fig. 9B). Trastuzumab alone did not induce apoptosis in NCI-N87 cells even after a two-fold increase in dose (data not shown). The effect of 1E11 was confirmed in the BT-474 cell line, where it showed a similar effect as a single agent and in combination with trastuzumab (Fig. 9C). To confirm that the cytotoxic effect of 1E11 is mediated by the induction of apoptosis, the activity of caspase-3 and -7 was examined in NCI-N87 cells. Caspase-3/7 activity increased slightly after 24 h of treatment with

Figure 9. 1E11 induces apoptosis in combination with trastuzumab

A, NCI-N87 cells were treated with antibodies for 48 h, stained with propidium iodide (PI), and DNA content was measured by flow cytometry. The relative cell populations in sub-G1 (subdiploid), G1, S, and G2-M phases are shown. **B**, NCI-N87 and **C**, BT-474 cells were treated with antibodies for 48 h and stained with annexin V-phycoerythrin (PE) and PI. Cell death was assessed by flow cytometry. The percentage of cells staining positive for annexin V (early apoptosis) and annexin V and PI (late apoptosis) are shown. **D**, NCI-N87 cells were treated with antibodies for 24 h and caspase-3/7 activity was analyzed.


A
NCI-N87
Cell population (%)
120
100
80
60
40
20
0
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11
G2/M
S
G0/G1
SubG1
B
NCI-N87
Apoptotic fraction (%)
35
30
25
20
15
10
5
0
Late apoptosis
Early apoptosis
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11
C
BT-474
Apoptotic fraction (%)
10
9
8
7
6
5
4
3
2
1
0
Late apoptosis
Early apoptosis
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11
D
NCI-N87
Capase-3/7 activity (fold)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11

trastuzumab as a single agent, whereas a 1.6-fold increase was observed in response to combination treatment with 1E11 (Fig. 9D). These results indicate that 1E11 exhibits antiproliferative activity by inducing apoptotic cell death, and its apoptotic activity is further increased in combination with trastuzumab in NCI-N87 gastric cancer cells.

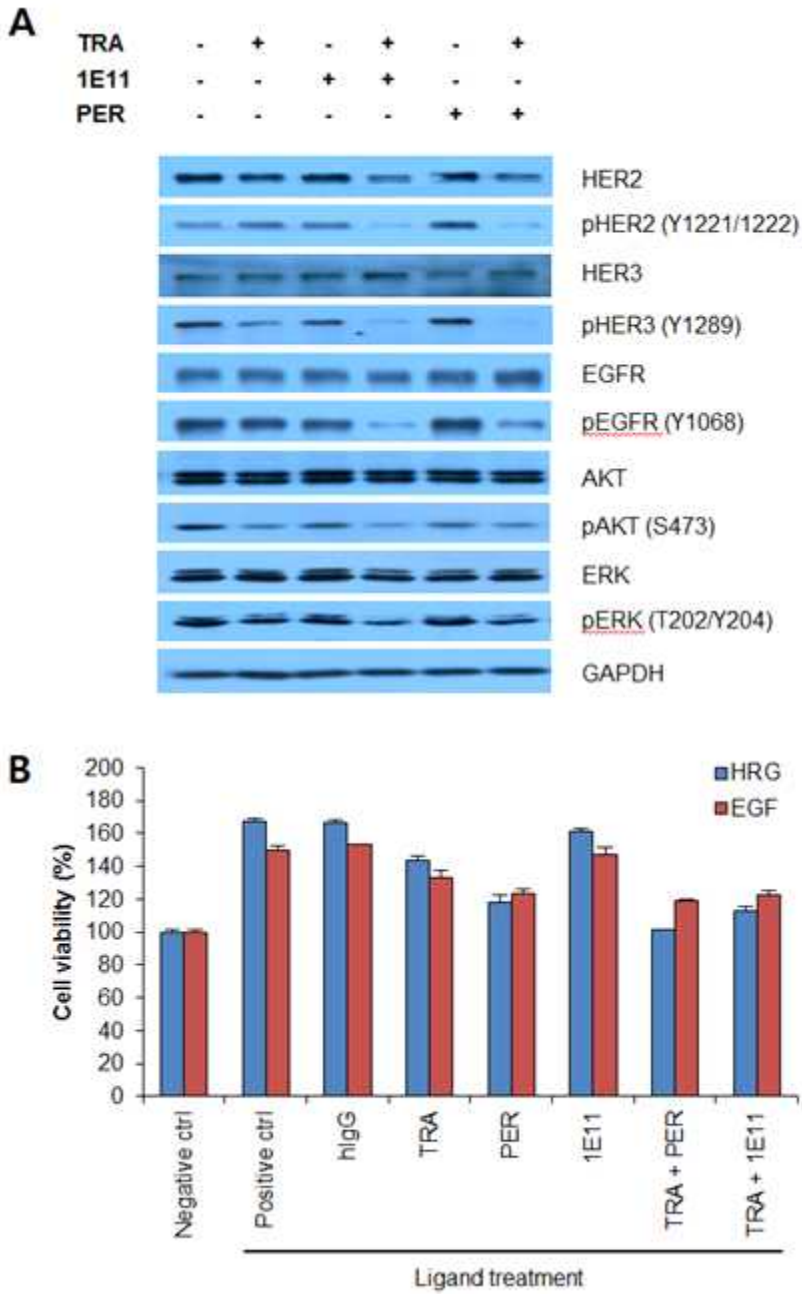
1E11 inhibits ErbB family signaling and ligand-induced cell proliferation in combination with trastuzumab

To elucidate the mechanism underlying the antiproliferative activity of 1E11 in combination with trastuzumab, the levels of the HER2 protein and related signaling molecules were measured. Combination treatment with 1E11 and trastuzumab or with pertuzumab and trastuzumab reduced total HER2 and phosphorylated HER2 levels (Fig. 10A). Combination treatment decreased the levels of activated HER3 and EGFR without affecting total protein levels and significantly downregulated the activated form of the HER2 downstream factors AKT and ERK while the total protein levels remained unchanged. These results suggest that 1E11 in combination with trastuzumab inhibits the activity of ErbB family proteins and suppresses downstream signaling.

To evaluate the significance of ErbB family receptor signaling in the response of HER2-overexpressing gastric cancer cells to antibody

Figure 10. 1E11 inhibits HER2 downstream signaling and heterodimerization-induced cell proliferation

A, NCI-N87 cells were treated with 10 mg/mL of antibodies for 24 h under normal growth conditions, and changes in HER2 and HER3 downstream signaling were monitored by western blotting. Phosphorylated proteins were detected using antibodies against well-characterized activation site residues. **B**, Cell growth inhibition in the presence of HRG1 or EGF was examined. Cell proliferation was assessed 3 days after the indicated treatments. Data points represent the mean ± SD (n = 3).


A
TRA - + - + - +
1E11 - - + + - -
PER - - - - + +
HER2
pHER2 (Y1221/1222)
HER3
pHER3 (Y1289)
EGFR
pEGFR (Y1068)
AKT
pAKT (S473)
ERK
pERK (T202/Y204)
GAPDH
B
Cell viability (%)
200
180
160
140
120
100
80
60
40
20
0
HRG
EGF
Negative ctrl
Positive ctrl
hIgG
TRA
PER
1E11
TRA + PER
TRA + 1E11
Ligand treatment

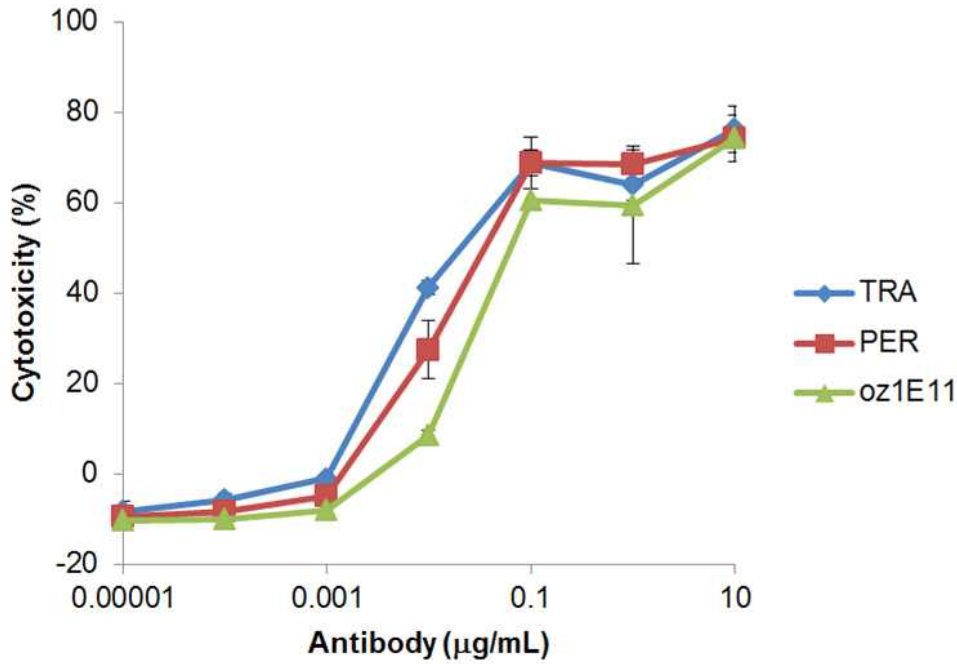
treatment, the antiproliferative activity of antibodies was analyzed under ligand-induced dimerization conditions. The induction of cell proliferation by HRG1 and EGF in NCI-N87 cells (Fig. 10B) was not inhibited by trastuzumab or 1E11 as a single agent, whereas pertuzumab inhibited ligand-induced cell proliferation as previously reported (Nahta et al., 2004). Combination treatment with 1E11 and trastuzumab inhibited ligand-induced proliferation to a similar level as the combination of trastuzumab and pertuzumab, suggesting that 1E11 in combination with trastuzumab could compensate for the effect of pertuzumab. These results suggested that in addition to the inhibition of ErbB family dimerization, other mechanisms are involved in the effect of combination treatment with 1E11 and trastuzumab on the inhibition of cell proliferation in HER2-overexpressing gastric cancer cells.

The antiproliferative activity of optimized 1E11 antibody (oz1E11) is dependent on HER2 expression

The oz1E11 antibody was developed by humanization and affinity maturation of 1E11. The binding affinity of oz1E11 for the ECD of HER2 (monomer) was 1.9 nM as determined by SPR, whereas that of the parental 1E11 antibody was 23 nM. The ADCC activity of oz1E11 was comparable to that of trastuzumab or pertuzumab in the lactate dehydrogenase (LDH) release assay performed using SK-BR-3 cells and PBMCs (Fig. 11) (Arnould et al.,

Figure 11. oz1E1 mediates antibody-dependent cellular cytotoxicity of SK-BR-3

The antibody-dependent cellular cytotoxicity of antibodies was measured using an assay that detects LDH released from lysed cells. Peripheral blood mononuclear cells were used as effector cells and HER2 overexpressing SK-BR-3 cells were used as target cells with 1:50 target:effector ratio. Cytotoxicity was expressed as the mean ± SD (n = 3).


100
80
60
40
20
0
-20
Cytotoxicity (%)
0.00001
0.001
0.1
10
Antibody (µg/mL)
TRA
PER
oz1E11

2006).

The antiproliferative activity of oz1E11 was examined in seven gastric cancer and two breast cancer cell lines. The oz1E11 antibody showed moderate antiproliferative activity as a single agent in HER2-overexpressing gastric cancer and breast cancer cells (Fig. 12), whereas in combination with trastuzumab, its antiproliferative activity was superior to that of pertuzumab or trastuzumab alone or in combination in HER2-overexpressing NCI-N87 and OE-19 gastric cancer cell lines (Fig. 13). Changes in cell viability in response to oz1E11 were correlated with HER2 levels in treated cells (Fig. 14), suggesting that the antiproliferative activity of oz1E11, both as a single agent and in combination with trastuzumab, is dependent on HER2 expression. The oz1E11 antibody showed high efficacy against HCC-202 breast cancer cells, which are HER2-positive but trastuzumab-resistant, whereas it showed little activity against other trastuzumab-resistant cell lines including HCC-1954 (Fig. 15).

The antiproliferative activity of oz1E11 was confirmed *in vivo* in NCI-N87 and OE-19 xenograft models. Tumors were treated when reaching a volume of 200 mm^3 or 500 mm^3 to evaluate the effect of the antibody on TGI and regression. Combination treatment with oz1E11 and trastuzumab showed superior antitumor activity to that of each agent alone in both xenograft models. In the *in vivo* efficacy study using NCI-N87 cells, pertuzumab showed increased antitumor activity when used in combination with trastuzumab, which was

different from the results of the *in vitro* study. Both oz1E11 and pertuzumab caused complete regression of NCI-N87 xenograft tumors when used in combination with trastuzumab (Fig. 16) However, in the OE-19 xenograft model, only the combination of oz1E11 and trastuzumab completely inhibited tumor growth, and pertuzumab and trastuzumab in combination caused partial inhibition of tumor growth (Fig. 17). Isolated OE-19 and NCI-N87 xenograft tumors were subjected to the TUNEL assay, which showed a higher number of apoptotic cells in tissues treated with oz1E11 that was further increased in response to combination treatment with oz1E11 and trastuzumab (Fig. 18).

Figure 12. oz1E11 shows antiproliferative activity in HER2-overexpressing gastric and breast cancer cells

Human gastric and breast cancer cells were treated with 5 mg/mL of antibodies for 3 or 4 days and cell viability was determined (*upper panel*). The expressions of HER2, EGFR, and HER3 in 20 mg of total cell lysate were determined by western blotting (*bottom panel*). Cell viability was expressed as the mean ± SD (n = 3), and the 100% point was defined as the absorbance of the untreated well.

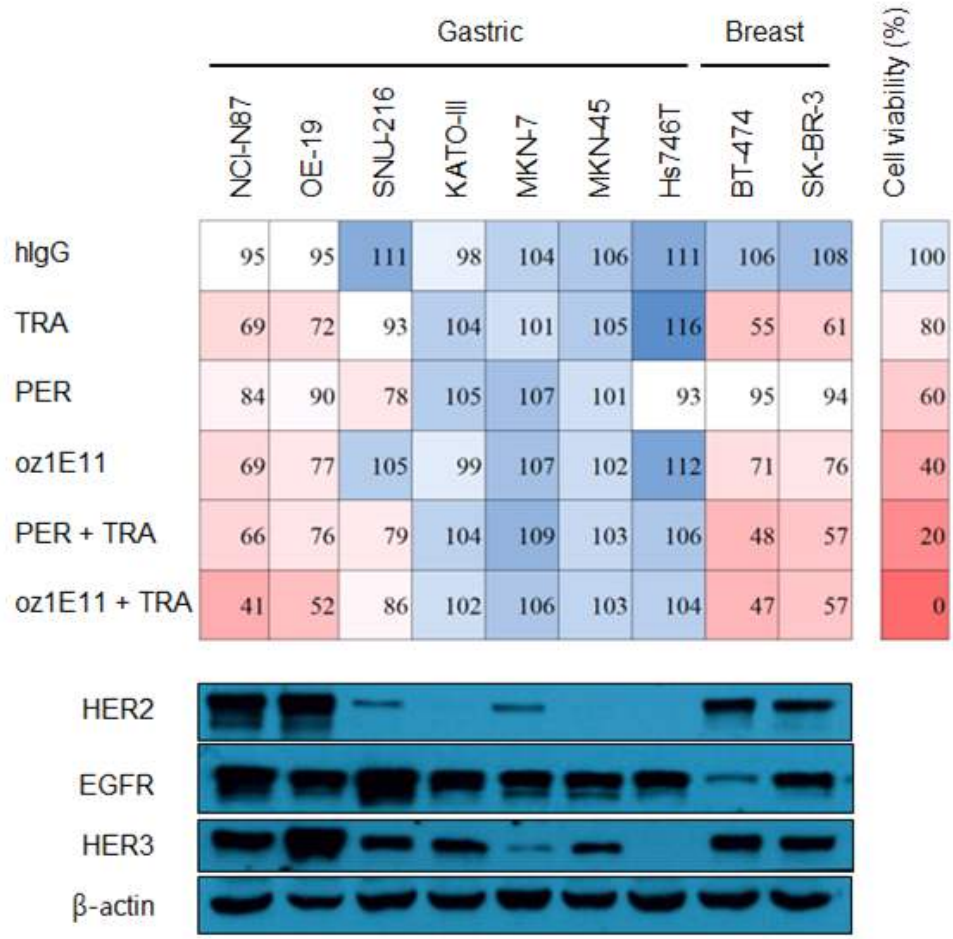

Gastric
Breast
Cell viability (%)
NCI-N87
OE-19
SNU-216
KATO-III
MKN-7
MKN-45
Hs746T
BT-474
SK-BR-3
hIgG
95 95 111 98 104 106 111 106 108
100
TRA
69 72 93 104 101 105 116 55 61
80
PER
84 90 78 105 107 101 93 95 94
60
oz1E11
69 77 105 99 107 102 112 71 76
40
PER + TRA
66 76 79 104 109 103 106 48 57
20
oz1E11 + TRA
41 52 86 102 106 103 104 47 57
0
HER2
EGFR
HER3
β-actin

Figure 13. oz1E11 shows synergistic antiproliferative activity in combination with trastuzumab in HER2-overexpressing gastric cancer

Dose-effect curves of antibodies in NCI-N87 (**A**) and OE-19 (**B**) cells are shown. Cell viability was expressed as the mean ± SD (n = 3), and the 100% point was defined as the absorbance of the untreated well.

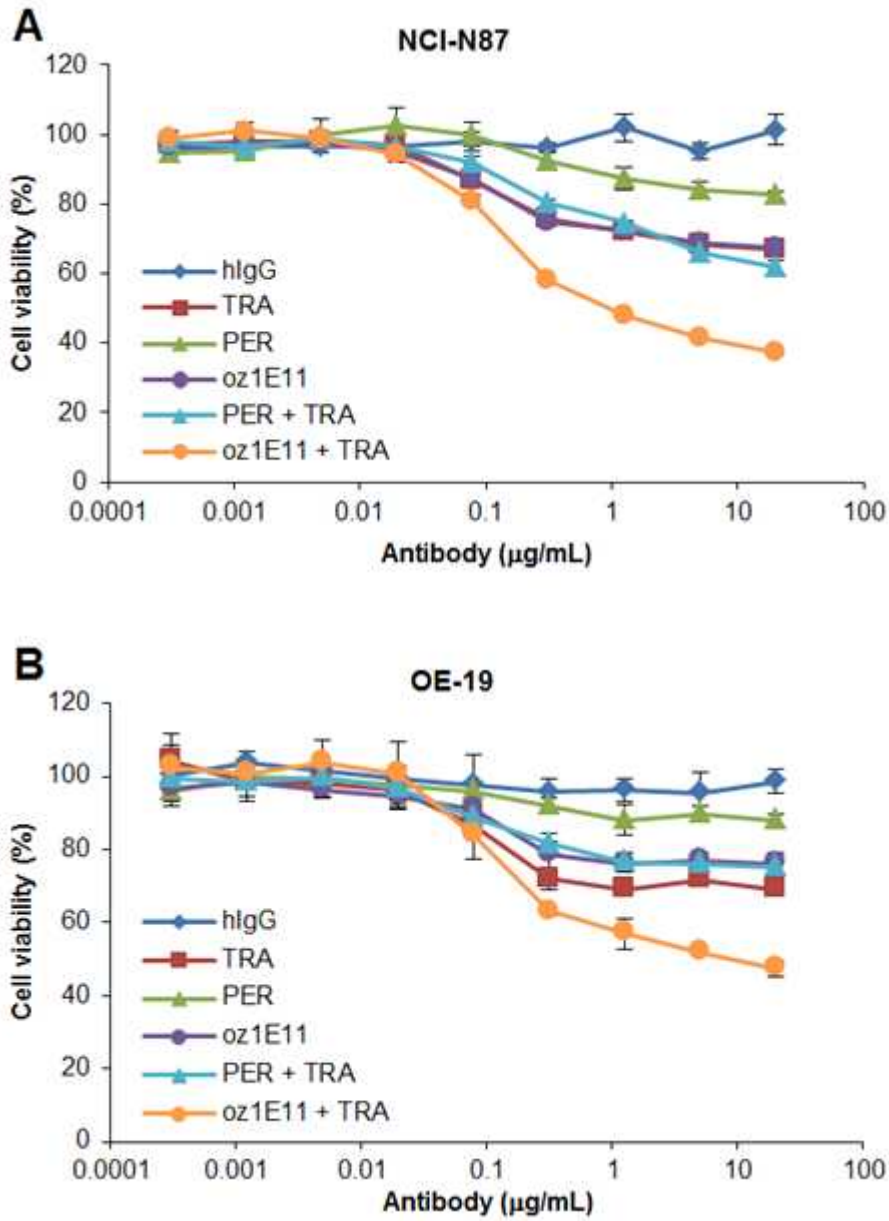

A
NCI-N87
Cell viability (%)
120
100
80
60
40
20
0
hIgG
TRA
PER
oz1E11
PER + TRA
oz1E11 + TRA
0.0001
0.001
0.01
0.1
1
10
100
Antibody (µg/mL)
B
OE-19
Cell viability (%)
120
100
80
60
40
20
0
hIgG
TRA
PER
oz1E11
PER + TRA
oz1E11 + TRA
0.0001
0.001
0.01
0.1
1
10
100
Antibody (µg/mL)

Figure 14. Expression of HER2 and EGFR in gastric and breast cancers

HER2 (**A**) and EGFR (**B**) expression levels were determined by flow cytometric analysis of 100,000 cells stained with 1 mg of trastuzumab or cetuximab for the detection of HER2 and EGFR, respectively. Control levels were determined with secondary antibody. Data points are the mean ± SD (n = 2) of the mean fluorescence intensity (MFI).

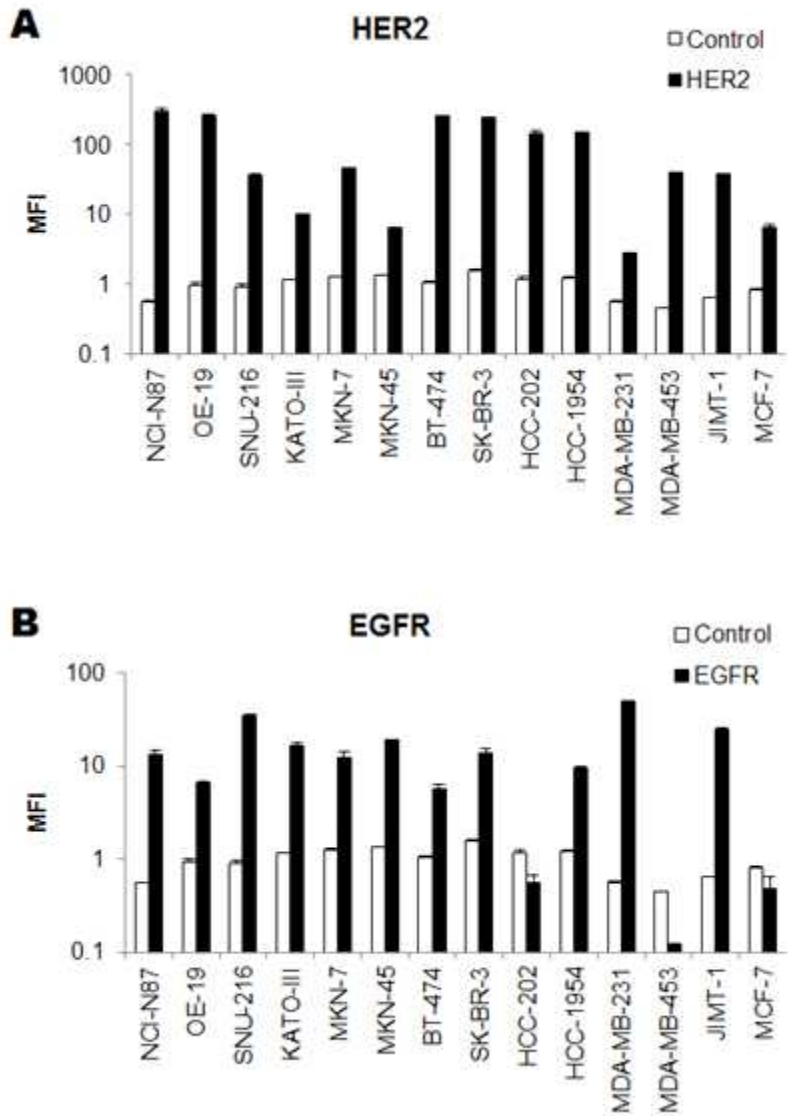

A
HER2
Control
HER2
MFI
1000
100
10
1
0.1
NCI-N87
OE-19
SNU-216
KATO-III
MKN-7
MKN-45
BT-474
SK-BR-3
HCC-202
HCC-1954
MDA-MB-231
MDA-MB-453
JIMT-1
MCF-7
B
EGFR
Control
EGFR
MFI
100
10
1
0.1
NCI-N87
OE-19
SNU-216
KATO-III
MKN-7
MKN-45
BT-474
SK-BR-3
HCC-202
HCC-1954
MDA-MB-231
MDA-MB-453
JIMT-1
MCF-7

Figure 15. oz1E11 shows antiproliferative activity in trastuzumab-resistant breast cancer cells

A, Human breast cancer cells were treated with 5 mg/mL of antibodies for 3 or 4 days and cell viability was determined. **B**, A dose-effect curve of antibodies in HCC-202 cells is shown. Cell viability was expressed as the mean ± SD (n = 3), and the 100% point was defined as the absorbance of the untreated well.

A

	BT-474	SK-BR-3	HCC-202	HCC-1954	MDA-MB-231	MDA-MB-453	JIMT-1	MCF-7	Cell viability (%)
hIgG	106	108	105	105	98	99	101	95	100
TRA	55	61	93	108	96	73	99	102	80
PER	95	94	93	96	97	90	96	98	60
oz1E11	71	76	77	100	92	87	99	97	40
PER + TRA	48	57	109	95	98	72	101	97	20
oz1E11 + TRA	47	57	53	100	96	67	102	97	0

B

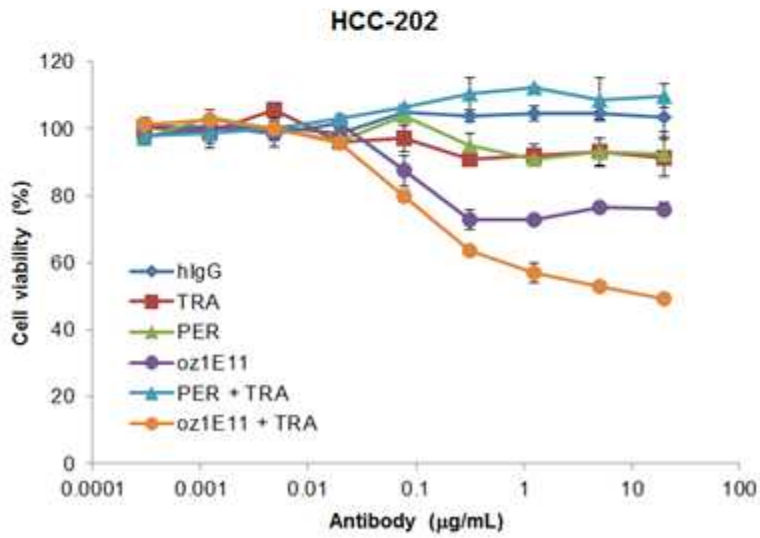
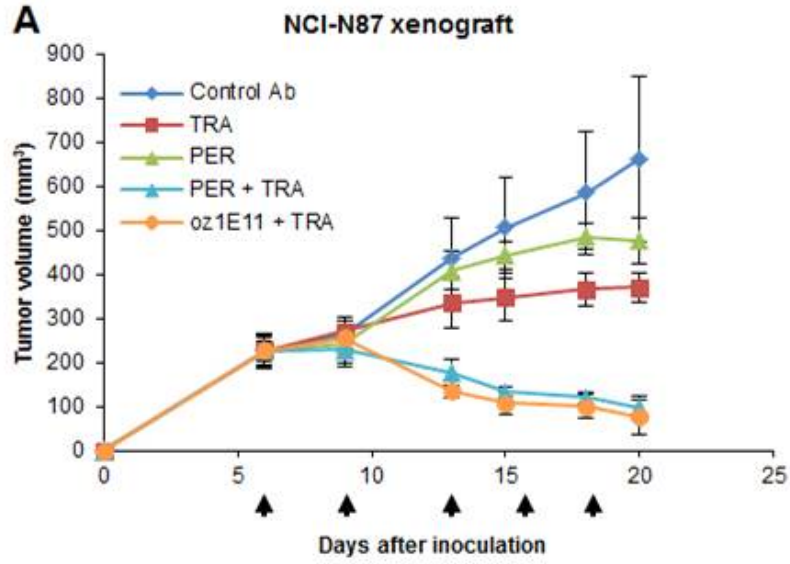


Figure 16. oz1E11 causes tumor regression in NCI-N87 gastric tumor xenograft models in combination with trastuzumab

A, NCI-N87 cells were inoculated into mice (n = 6 mice/group each), and treatment with 10 mg/kg of antibody was started when tumor volumes reached approximately 200 mm^3. For combination treatment, 10 mg/kg of each antibody was administered. **B**, NCI-N87 (n = 5 mice/group) cells were inoculated into mice and antibody treatments were started when tumor volumes reached approximately 500 mm^3. Mice received a dose of 20 mg/kg for single agent treatment and 10 mg/kg of each antibody for combination treatment. Administration days are indicated by arrows. Tumor volume (mm^3) was expressed as the mean ± SD. Statistically significant differences were determined by Student's t test. *, $P < 0.05$ versus the pertuzumab and trastuzumab combination.


A
NCI-N87 xenograft
Control Ab
TRA
PER
PER + TRA
oz1E11 + TRA
Tumor volume (mm³)
Days after inoculation

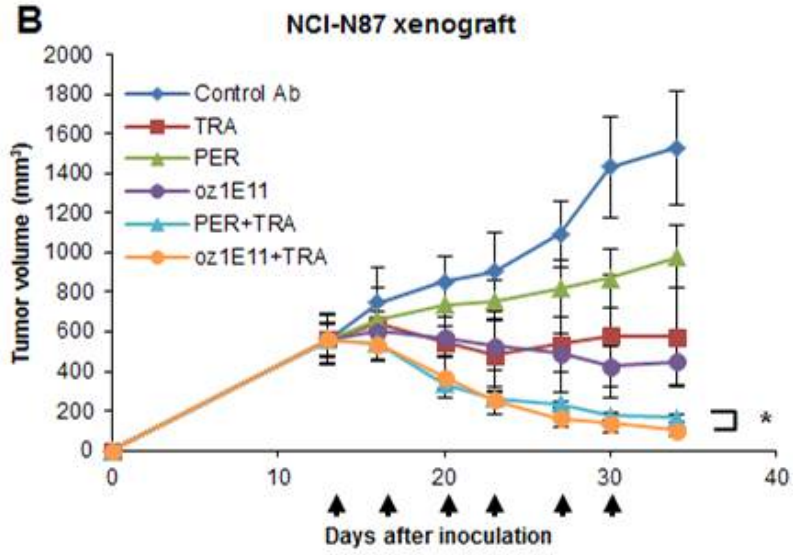
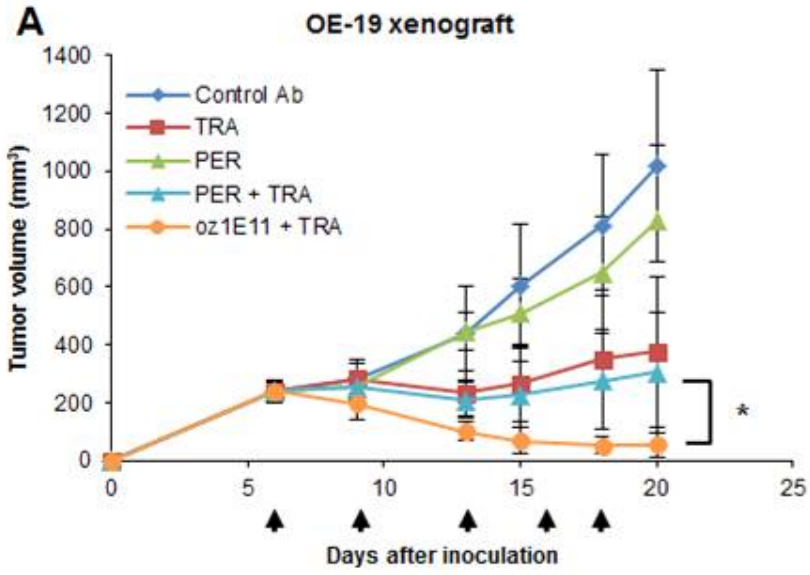

B
NCI-N87 xenograft
Control Ab
TRA
PER
oz1E11
PER+TRA
oz1E11+TRA
Tumor volume (mm³)
Days after inoculation
*

Figure 17. oz1E11 causes tumor regression in OE-19 gastric tumor xenograft models in combination with trastuzumab

A, OE-19 cells were inoculated into mice (n = 6 mice/group each), and treatment with 10 mg/kg of antibody was started when tumor volumes reached approximately 200 mm^3. For combination treatment, 10 mg/kg of each antibody was administered. **B**, OE-19 (n = 3 mice/group) cells were inoculated into mice and antibody treatments were started when tumor volumes reached approximately 500 mm^3. Mice received a dose of 20 mg/kg for single agent treatment and 10 mg/kg of each antibody for combination treatment. Administration days are indicated by arrows. Tumor volume (mm^3) was expressed as the mean ± SD. Statistically significant differences were determined by Student's t test. *, $P < 0.05$ versus the pertuzumab and trastuzumab combination.


A
OE-19 xenograft
Tumor volume (mm³)
1400
1200
1000
800
600
400
200
0
Control Ab
TRA
PER
PER + TRA
oz1E11 + TRA
*
0
5
10
15
20
25
Days after inoculation

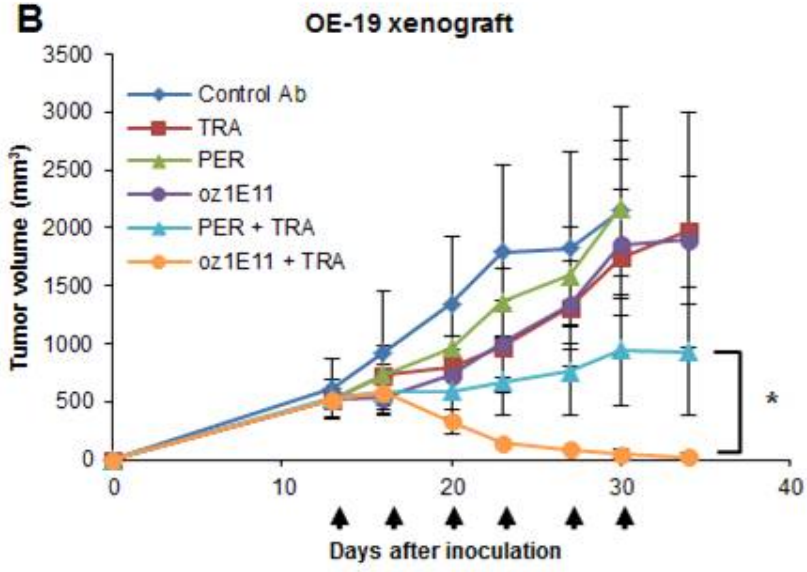
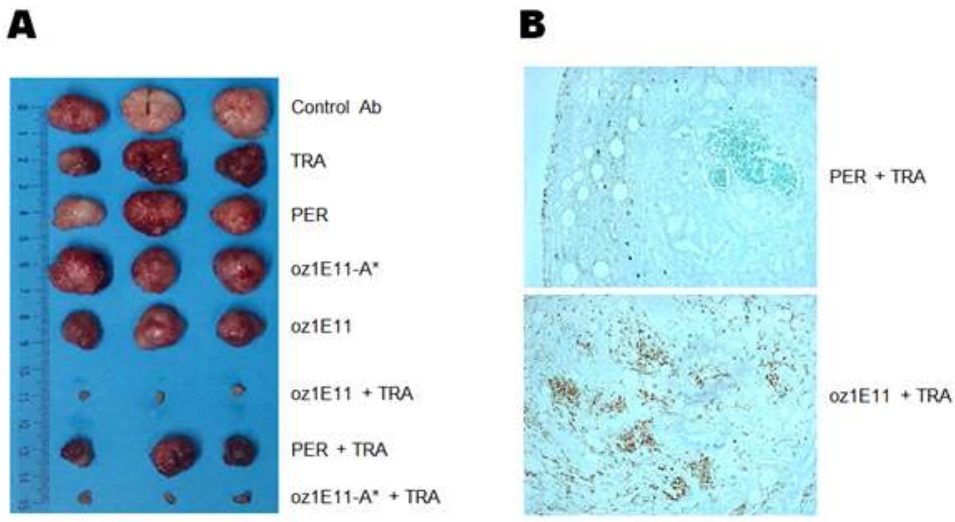

B
OE-19 xenograft
Tumor volume (mm³)
3500
3000
2500
2000
1500
1000
500
0
Control Ab
TRA
PER
oz1E11
PER + TRA
oz1E11 + TRA
*
0
10
20
30
40
Days after inoculation

Figure 18. Apoptotic activity of oz1E11 in xenograft models

A, Tumor masses were isolated after the OE-19 xenograft study described in Figure 17B that antibody treatments were started when tumor volumes reached approximately 500 mm^3. TUNEL assay was followed using these OE-19 cell mass (**B**). **C**, Another TUNEL assay was conducted with isolated tumor masses from NCI-N87 xenograft study that antibody treatments were started when tumor volumes reached approximated 500mm^3 (Fig. 16B). *oz1E11-A is another optimized antibody derived from 1E11.


A
Control Ab
TRA
PER
oz1E11-A*
oz1E11
oz1E11 + TRA
PER + TRA
oz1E11-A* + TRA
B
PER + TRA
oz1E11 + TRA
C
PER + TRA
oz1E11 + TRA

Discussion

HER2 targeted therapy has shown promising results in the treatment of gastric cancer, and trastuzumab has been approved for the treatment of patients with HER2-overexpressing gastric cancer. However, despite encouraging clinical results obtained with trastuzumab, the development of more potent targeted therapies for HER2-positive gastric cancer is necessary to increase overall survival rates (Bang et al., 2010). Combination of non-competing antibodies targeting receptor proteins can increase antitumor activity *in vitro* and *in vivo* (Kamat et al., 2008; Pedersen et al., 2010; Tvorogov et al, 2010; and Zhang et al., 2010). Pertuzumab is the first approved antibody to be used in combination for dual targeting of the same protein in the treatment of metastatic breast cancer (Baselga et al., 2012). In the present study, efforts to identify a superior therapeutic antibody for HER2-overexpressing gastric cancer resulted in the development of 1E11. 1E11 inhibits HER2-overexpressing gastric cancer cell growth in *in vitro* and *in vivo* models in combination with trastuzumab, enhancing its apoptotic activity in a synergistic manner. Unlike pertuzumab and trastuzumab, 1E11 and trastuzumab bind to the same sub-domain (IV) of HER2. To the best of our knowledge, this is the first antibody combination targeting the same sub-domain of the HER2 protein with potential therapeutic application in the treatment of gastric cancer.

HER2/HER3 heterodimerization is one of the most common mechanisms triggering aberrant HER2 signaling in breast cancer (Baselga and Swain, 2009 and Olayioye et al., 2000), and pertuzumab has shown antitumor activity through the inhibition of HER2/HER3 dimerization in non-small cell lung cancer and breast cancer (Lee-Hoeflich et al., 2008 and Sakai et al., 2007). The NCI-N87 cell line has previously been used as a model system for research on HER2-overexpressing gastric cancer (Kim et al., 2008; Patel et al., 2009; Tanner et al., 2005; and Yamashita-Kashima et al., 2011). In the present study, NCI-N87 cell proliferation was increased by the HER2 heterodimerizing ligands HRG1 and EGF, and treatment with pertuzumab efficiently inhibited ligand-induced cell proliferation as demonstrated previously (Fig. 10B). 1E11 in combination with trastuzumab inhibited ligand-induced cell proliferation to a similar level as the pertuzumab/trastuzumab combination, suggesting that the effect of 1E11 in combination with trastuzumab is mediated by the inhibition of HER2 heterodimerization. Pertuzumab did not show significant antiproliferative activity as a single agent or in combination with trastuzumab in NCI-N87 and OE-19 cells. By contrast, the antiproliferative activity of 1E11 increased when used in combination with trastuzumab. These results suggest that blocking HER2 heterodimerization with EGFR or HER3 is not fully sufficient to induce cell death in HER2-overexpressing gastric cancer.

One of the hallmarks of cancer is resistance to apoptosis (Hanahan and Weinberg, 2011 and Susnow et al., 2009), and the induction of

apoptosis is an important antitumor mechanism of therapeutic antibodies (Ben-Kasus et al., 2007). The CD20-targeting antibody rituximab activates caspase-3 through a mechanism involving Src family kinases, and the EGFR-targeting antibody cetuximab upregulates the pro-apoptotic protein Bax and downregulates the anti-apoptotic protein Bcl-2 (Hofmeister et al., 2000 and Huang et al., 1999). HER2 suppresses pro-apoptotic Bad and Bim through AKT (Datta et al., 1997 and Tanizaki et al., 2011), and AKT regulates several members of the forkhead family of transcription factors related to apoptosis (Chakrabarty et al., 2013 and Real et al., 2005). Recently, translocation of HER2 to mitochondria was shown to contribute to trastuzumab resistance by decreasing the activity of cytochrome c oxidase in a HER2-dependent manner (Ding et al., 2012). 1E11 significantly induced apoptosis in NCI-N87 cells as single agent and in combination with trastuzumab (Fig. 9B and 9C), and the apoptotic activity of 1E11 was confirmed in xenograft model (Fig. 18). As reported earlier (Nahta et al., 2004) and confirmed here, the apoptotic activity of trastuzumab is limited. Therefore, the synergistic increase in antitumor activity by the combination of 1E11 and trastuzumab could be attributed to the apoptotic activity of 1E11. The apoptotic cancer cell death induced by 1E11 and trastuzumab in combination could be mediated by the downregulation of HER2 and inhibition of PI3K-AKT signaling. 1E11 and trastuzumab combination treatment increased effector caspase-3/7 activity confirming that the apoptotic activity of 1E11 (Fig. 9D). Trastuzumab alone induces cell

cycle arrest in BT-474 cells as previously reported (Brockhoff et al., 2007). However, in the present study, trastuzumab and 1E11 did not cause significant cell cycle arrest as single agents or in combination in NCI-N87 cells.

Despite the important roles of sub-domains II and IV of the HER2 extracellular region in ErbB family dimerization activation of downstream signaling pathways associated with cell growth (Burgess et al., 2003), the exact role of sub-domain IV remains unknown. It has been reported that trastuzumab disrupts HER2/HER3 heterodimer-formation under ligand-independent conditions (Junttila et al., 2009); however, the direct interaction of HER2 with non-ErbB family members such as CD44, MUC4, and IGF-1R plays a role in tumor development and trastuzumab resistance (Balana et al., 2001; Bourguignon et al., 1997; and Nagy et al., 2005). Pertuzumab inhibits HER2 and IGF-1R dimerization in trastuzumab-resistant breast cancer cells, although it does not significantly inhibit cell growth (Nahta et al., 2005). 1E11, in combination with trastuzumab, inhibited ligand-induced cell proliferation to a similar level as pertuzumab, despite the fact that 1E11 binds to a non-overlapping epitope in sub-domain IV that is not associated with the dimerization arm (Fig. 7). Similar to previously reported antibody combinations, the 1E11 and trastuzumab combination could inhibit HER2 dimerization with other non-ErbB2 family members, resulting in cancer cell apoptosis and/or downregulation of HER2 (Ben-Kasus et al., 2009; Kamat et al., 2008; and Pedersen et al., 2010). oz1E11 had antiproliferative effects against

HCC-202, a HER2-positive but trastuzumab-resistant breast cancer cell line, although the mechanism by which oz1E11 overcomes trastuzumab resistance in this particular cell line remains unclear. Further investigation is necessary to improve our understanding of the mode of action of oz1E11 (Fig. 15).

In conclusion, 1E11 showed synergistic antitumor activity in combination with trastuzumab in HER2-positive human gastric cancer *in vitro* and *in vivo*. The antitumor activity of 1E11 was mediated by its apoptotic activity and the inhibition of HER2 homo-and hetero-dimerization downstream signaling. To the best of our knowledge, 1E11 and trastuzumab is the first antibody combination targeting the same sub-domain of HER2. This antibody combination could be a useful tool to improve our understanding of the biochemical properties of HER2 and a novel potent therapeutic strategy for the treatment of patients with HER2-overexpressing gastric and breast cancer.

CHAPTER II

Optimization of a HER2 targeting mouse monoclonal antibody 1E11 for therapeutic development using human germline genes and targeted randomization of CDR-L3

Abstract

The mouse monoclonal antibody 1E11, targeting epidermal growth factor receptor 2 (HER2), shows synergistic antitumor activity in HER2-overexpressing gastric cancer cells when used in combination with trastuzumab. In the present study, I optimized this antibody for therapeutic development. First, the CDRs of the mouse antibody were grafted onto human germline immunoglobulin variable genes. No difference in affinity and biological activity was observed between chimeric 1E11 (ch1E11) and humanized 1E11 (hz1E11). Next, affinity maturation of hz1E11 was performed by the randomization of CDR-L3 and H3 residues followed by stringent biopanning selection. It was observed that milder selection pressure favored the selection of more diverse clones, whereas higher selection stringency resulted in the convergence of the panning output to a smaller number of clones with improved affinity. The clone 1A12 had 4 amino acid substitutions in CDR-L3, and showed a 10-fold increase in affinity compared to the parental clone and increased potency in the antiproliferative activity assay with HER2-overexpressing gastric cancer cells. The clone 1A12 inhibited the tumor growth of NCI-N87 and OE-19 xenograft models with similar efficacy to trastuzumab as a single agent, and the combination treatment of 1A12 and trastuzumab completely removed the established tumors. These results suggest that humanized and affinity matured monoclonal antibody

1A12 is a highly optimized molecule for future therapeutic development against HER2-positive tumors in combination with trastuzumab.

Introduction

Monoclonal antibodies are mainstream treatments in oncology and autoimmune diseases and expected to play an important roles in the future of diseases treatment (Chan and Carter, 2010; Weiner et al., 2010). More than thirty recombinant antibodies are currently approved by the US FDA, of which the approximately a half are anti-cancer antibodies. Gastric cancer is one of the most common cancers, and is the third leading cause of cancer death worldwide (Ferlay et al., 2012). In gastric cancer, overexpression of EGFR, HER2, and HER3 is correlated with poor prognosis (Garcia et al., 2003 and Hayashi et al., 2008). Recently, the HER2 targeting monoclonal antibody trastuzumab was approved for the treatment of HER2-positive metastatic gastric and gastroesophageal junction cancer based on ToGA clinical trial (Bang et al., 2010).

Particular combinations of noncompetitive antibodies targeting the same receptor increase antitumor activity *in vitro* and *in vivo*. Combination of HER2 targeting antibodies, trastuzumab and pertuzumab, shows increased efficacy in HER2-overexpressing breast cancers (Nahta et al., 2004). The benefits of the pertuzumab and trastuzumab combination were further demonstrated in preclinical and clinical trials (Baselga et al., 2010 and Scheuer et al., 2009). Other HER2-targeting antibodies showing better efficacy in combination than as single agents have been reported, and have shown consistent

downregulation of HER2 levels and beneficial combination effects in mouse models (Ben-Kasus et al., 2009). The increased efficacy of antibody combinations has also been demonstrated with EGFR-targeting antibodies (Kamat et al., 2008; Pedersen et al., 2010) and VEGFR3-targeting antibodies (Tvorogov et al, 2010).

Therapeutic antibodies typically are extensively engineered, so that they possess desirable biological and physicochemical properties such as low immunogenicity, high affinity and specificity, optimal effector functions, and good solubility and stability (Igawa et al., 2012). Especially, antibody humanization and affinity maturation are two of the most frequently applied engineering processes during the development of therapeutic antibody candidates. Immunogenicity of therapeutic antibody limits the clinical utility and efficacy by production of anti-drug antibodies (Hwang and Foote, 2005), and the humanization of the antibodies from mouse or other species is now a standard procedure for the development of therapeutic antibodies. Many humanization methods have been developed that employ either rational or empirical approaches (Almagro and Fransson, 2008). The complementarity-determining region (CDR) grafting approach as a method to overcome the human anti-chimeric antibody (HACA) response (Jones et al., 1986) is a well-established humanization method. However, direct grafting of murine CDRs onto a human framework acceptor sequence often results in a loss of affinity, so back-mutations of framework region residues (Vernier zone residues; see (Almagro and Fransson, 2008)) supporting the structure of CDR

loops is often necessary (Foote and Winter, 1992). For *in vitro* affinity maturation, three diversification approaches are typically used: random mutagenesis by e.g. error-prone PCR, randomization of targeted residues, and chain shuffling. In the targeted randomization approach, CDRs are the target for the randomization in most cases because somatic hypermutation has evolved to favor mutations in CDRs of antibodies (Wu et al., 2003) and CDR-H3 and CDR-L3 tend to dominate the antibody-antigen interaction (Sundberg and Mariuzza, 2002). One of the main problems associated with the targeted randomization is selecting the positions that are not essential for the antigen binding, but can enhance the affinity when optimal substitution of amino acid is made. Alanine scanning can help deciding which residues to randomize, especially when CDRs are long. Sometimes, alanine mutation itself increases the affinity of antibodies (Kawa et al., 2011).

Previously we developed a mouse antibody targeting HER2 (clone 1E11) that shows synergistic antitumor activity in combination with trastuzumab in HER2 overexpressing gastric cancer cell lines (in press). In this study, we report the optimization of 1E11 for therapeutic development, by CDR grafting to human germline immunoglobulin variable genes and affinity maturation through targeted randomization of CDR-H3 and CDR-L3. The optimized 1E11 antibody (clone 1A12) shows synergistic antitumor activity in HER2-positive gastric cancer xenograft models in combination with trastuzumab. It was observed that for the clone 1E11, human

germline variable genes are suitable acceptors for humanization without affinity reduction, and randomization of non-essential residues in CDR-L3 is enough to improve the affinity by more than 10-fold.

Materials and Methods

Cell lines and materials

NCI-N87 cells were purchased from American Type Culture Collection (ATCC) and OE-19 cells were obtained from the European Collection of Cell Culture (ECACC). Cell culture media were RPMI-1640 supplemented with 10% fetal bovine serum (FBS), and antibiotics and cells were cultured at 37°C under 5% CO_2. Trastuzumab were produced by Genentech Incorporated (South San Francisco, CA, USA), and palivizumab was produced by MedImmune, LLC (Gaithersburg, MD, USA). ChromPure human IgG (Jackson Immunoresearch Lab, West Grove, PA, USA) was used as human IgG control antibody in *in vitro* assays. IgG antibodies were produced using the Freestyle 293 system (Invitrogen) and purified using protein-A chromatography (GE Healthcare). Endotoxin was removed with an Endotoxin removal kit (GenScript), and endotoxin levels were determined using an Endotoxin detection kit (GenScript). Recombinant proteins were produced as secretion proteins using the 293F system and purified using protein-A or Ni-NTA chromatography (Qiagen Inc.) for Fc-tagged and His-tagged proteins, respectively.

Alanine-scanning mutagenesis and Fab purification

Site-directed mutagenesis for alanine scanning was accomplished by PCR mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). Mutant Fab proteins were expressed and purified to evaluate the importance of each residue for antigen binding activity. In brief, *E.coli* DH5α cells, transformed with the pComb3X vector harboring mutant Fab genes, were grown at 28℃ in SB broth; expression was induced with 1 mM IPTG when the optical density (600 nm) of the culture reached 0.8. Cell pellets were resuspended in chilled extraction buffer (120 mM Tris pH 8.0, 0.3 mM EDTA, and 300 mM sucrose) and incubated on ice for 30 min for periplasmic extraction. Magnesium chloride (2.5 mM) was added to the clarified extract prior to immobilized metal ion affinity chromatography (IMAC) purification. Purified Fab proteins were used for ELISA binding assay and k_{off} analysis using surface plasmon resonance (SPR).

Affinity maturation

Humanized 1E11, cloned in pComb3X vector as Fab format, was utilized as template for overlap extension polymerase chain reaction (PCR) mutagenesis as described earlier (Barbas et al., 2001). The degenerate oligonucleotides 5'-CTGCCCGAAGGTCCAGGGKNNKNNKNNKNNCTGCTGGCAGTA

ATAAGTAGC-3' (reverse) and 5'-GTCTACTATTGTGCTAGA42S43S11S11S24S14S34STTCGACTAC TGGGGCCAGGG-3; (forward) were used for L-NNK and H-XXS library, respectively. N denotes A, C, G or T; K is G or T; and S is G or C. Numbered base positions indicate hand-mixed nucleotides which are composed of 70% of one base and 10% each of the other three bases; 70% frequency base is G, A, T, and C for 1, 2, 3, and 4, respectively. For H-2AA library, equimolar mixture of following forward mutagenic oligonucleotide were used: 5'-GTCTACTATTGTGCTAGANNKNNKGGTGGGACCGCCTCCTTC G A C - 3 ', 5'-GTCTACTATTGTGCTAGACACNNKNNKGGGACCGCCTCCTTCG A C T A C - 3 ', 5'-GTCTACTATTGTGCTAGACACCTGNNKNNKACCGCCTCCTTCG A C T A C T G G - 3 ', 5'-GTCTACTATTGTGCTAGACACCTGGGTNNKNNKGCCTCCTTC G A C T A C T G G G C - 3 ', 5'-GTCTACTATTGTGCTAGACACCTGGGTGGGNNKNNKTCCTTC GACTACTGGGGCCAG-3', and 5'-GTCTACTATTGTGCTAGACACCTGGGTGGGACCNNKNNKTTC GACTAC TGGGGCCAGGG-3'. After two round of overlap extension PCR, Fab library DNA with a randomized CDR was cut with SfiI, ligated into SfiI- digested phagemid vector pComb3X, and electrotransformed into *Escherichia coli* strain ER2537 (New England Biolabs, Beverly, MA).

High affinity binders were selected using soluble biotinylated HER2-ECD protein. Bead binders were pre-depleted by incubating the antibody phage library with 100 mL of pre-blocked Dynabeads M-280 Streptavidin (Invitrogen) for 1 h with gentle rotation at room temperature. Biotinylated HER2-ECD protein (100 nM - 0.1 pM) was added to the pre-depleted antibody library and incubated for 1 h with rotation at room temperature. Then 50 mL of blocked streptavidin-magnetic beads were added and incubated on the rotator for 15 min. Beads were washed 10 times with 1 mL of PBS-T buffer and twice with 1 mL of PBS-T. Remaining phages were eluted by incubating the beads with 1 mL of 100 mM triethylamine for 8 min then neutralized with 0.5 mL of 1M Tris, pH 7.4.

ELISA binding activity test

Antibodies were added to Costar 96-well half area plates (Corning) coated with 25 mg/mL of the antigen. After incubation at RT for 1 h, the plates were washed 3 times with TBST (50 mM Tris, pH7.6, 150 mM NaCl, 0.05% Tween 20) and incubated with goat anti-human antibody-HRP (Pierce) for IgG antibodies and rat anti-HA-Peroxidase (Roche) for Fab antibodies, respectively. Plates were washed 3 times, 3,3‘,5,5’-tetramethylbenzidine (TMB) peroxidase substrate (SurModics, Eden Prairie, USA) was added, and reactions were stopped by adding 1N sulfuric acid (DukSan, Seoul, Republic of Korea). Absorbance at 450-nm was measured using Victor X3 instrument.

Surface plasmon resonance (SPR) analysis

For k_{off} analysis of Fab proteins, HER2-ECD-His protein was immobilized onto CM5 sensor chip surface (GE Healthcare) using the amine coupling method at approximately 2,000 response units (RU). Purified Fab proteins were injected at 2 mg/mL concentration at 50 mL/min flow rate. For k_{off} analysis of IgG antibodies, goat anti-human IgG (ɣ) (Invitrogen) was immobilized onto a CM5 sensor chip using amine coupling, and antibodies were captured at 1 mg/mL for 4 min and stabilized for 5 min. Then, HER2-ECD-His protein was injected at 160 nM concentration. For affinity measurements, antibodies were captured at approximately 50 RU through goat anti-human IgG (ɣ). Then, HER2-ECD-His protein was injected at concentrations ranging from 0 to 640 nM. Sensorgrams were obtained at each concentration and evaluated using the BIAevaluation software. For epitope binning, IgG form of hz1E11 was immobilized onto separate CM5 sensor chip surfaces at approximately 1000 response units. HER2-ECD-His (320 nM) and antibodies (1 mg/mL) were sequentially added by binding for 4 min and stabilization for 5 min at 50 mL/min flow rate.

Cell viability assay

Cells were seeded in 96-well plates (Corning) in growth media containing 10% FBS and pre-cultured for 24 h. The cells were

treated with antibodies at the indicated concentrations and culture for 4 days. The WST reagent (DoGen, Daejeon, Republic of Korea) was used to measure cell viability. Relative cell viability was calculated by dividing the absorbance of each well by the mean absorbance of PBS-treated wells in each plate.

Xenograft study

Athymic nude female mice (Daehan Biolink, Korea) were injected subcutaneously in the left flank area with 5×10^6 of NCI-N87 or OE-19 cells in Matrigel (BDBiosciences). Tumors were allowed to grow to approximately 200 mm^3 in size, and mice were than randomized into groups. Animals received intraperitoneal administration of antibodies at the indicated doses twice weekly. Tumor volumes were calculated using the formula (L×W×W)/2, where "L" represents the larger tumor diameter and "W" represents the smallest tumor diameter.

All animal studies were conducted in accordance with the guidelines of the NIH's "Guide for Care and Use of Animals" and an approved protocol received by the company's Institution Animal Care and Use Committee.

Results

Humanization of 1E11 is conducted by CDR-grafting to human germline genes

To develop the humanized antibody, the VH and VL sequences of the murine 1E11 were compared with human germline V and J gene repertories using IMGT/V-QUEST analysis tools (Brochet et al., 2008). For the heavy chain, IGHV3-48*03 and IGHJ4*01 exhibited highest homology to the 1E11 counterparts, sharing 85% and 87% identity, respectively. For the light chain, human IGKV1-39*01 and IGKJ1*01 genes displayed identity of 80% and 81%, respectively. These human genes were selected as acceptor sequences for the grafting of the murine CDRs. Among "Vernier zone" which consists of residues in the framework region that are involved in the presentation of CDR structures by supporting the CDR loops (Foote and Winter, 1992 and Makabe et al., 2008), only one residue at position 49_H of heavy chain differed between murine and human sequences. Consequently, humanized 1E11, hz1E11, has only one murine residue in framework region (Fig. 19).

Binding activity of hz1E11 to extracellular region (ECD) of HER2 was equivalent to that of ch1E11 (Fig. 20A), and the affinities of trastuzumab, ch1E11 and hz1E11 were 3 nM, 23 nM, and 23 nM, respectively. We also confirmed that hz1E11 bound to sub-domain IV

Figure 19. Humanization of 1E11 by CDR grafting

Heavy chain (**A**) and light chain (**B**) amino acid sequence alignment of murine, human germline, and humanized 1E11. The CDRs, defined according to Kabat's nomenclature, are in bold (Kabat et al., 1991). Amino acids are numbered according to the Kabat numbering scheme. Colons represent common residues between murine and human germline.

A

```
                                                  31 32 33 34 35                                  50 51 52 52a 53 54 55 56 57 58 59 60
Murine 1E11       EVKLVESGGGLVQPGGSLKLSCAASGFTFS S Y T M S WVRQTPEKRLEWVA Y I S N G G G S T Y Y P
Human Germline    ::Q:::::::::::::::R:::::::::::: : : E : N ::::A:G:G::::S : : : S S : S T I : : A
Humanized 1E11    EVQLVESGGGLVQPGGSLRLSCAASGFTFS S Y T M S WVRQAPGKGLEWVA Y I S N G G G S T Y Y P

                  H61 H62 H63 H64 H65                                   95 96 97 98 99 100 100a 100b 101 102
Murine 1E11       D T V K G RFTISRDNAKNTLYLQMSSLKSEDTAMYYCAR H L G G T A S F D Y WGQGTIVTVSS
Human Germline    : S : : : ::::::::::::S:::::N::RA::::V:::::          D A : : : :::::L:::::
Humanized 1E11    D T V K G RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR H L G G T A S F D Y WGQGTLVTVSS
```

B

```
                                          24 25 26 27 28 29 30 31 32 33 34                  50 51 52 53 54 55 56
Murine 1E11       DIQMTQSPASQSASLGESVTITC L A S Q T I G T W L A WYQQKPGKSPQLLIY V A T S L A D GVPS
Human Germline    ::::::::S:L:::V:DR::::: R : : : S : S S Y : N ::::::::A:K:::: A : S : : Q S ::::
Humanized 1E11    DIQMTQSPSSLSASVGDRVTITC L A S Q T I G T W L A WYQQKPGKAPKLLIY V A T S L A D GVPS

                                          89 90 91 92 93 94 95 96 97
Murine 1E11       RFSGSGSGTKFSFKISSLQAEDFVSYYC Q Q L Y S T P W T FGGGTKLELK
Human Germline    :::::::::D:TLT:::::P:::AT::: : : S : : : : : : ::Q:::V:I:
Humanized 1E11    RFSGSGSGTDFTLTISSLQPEDFATYYC Q Q L Y S T P W T FGQGTKVEIK
```

Figure 20. Humanized 1E11 shows equivalent binding properties to parental antibody.

A, The binding activities of hz1E11 and ch1E11 to HER2-ECD protein were analyzed by ELISA. Trastuzumab (TRA) was used as the control antibody for the HER2 protein. **B**, The binding activity of antibodies was analyzed by ELISA using recombinant sub-domain proteins.

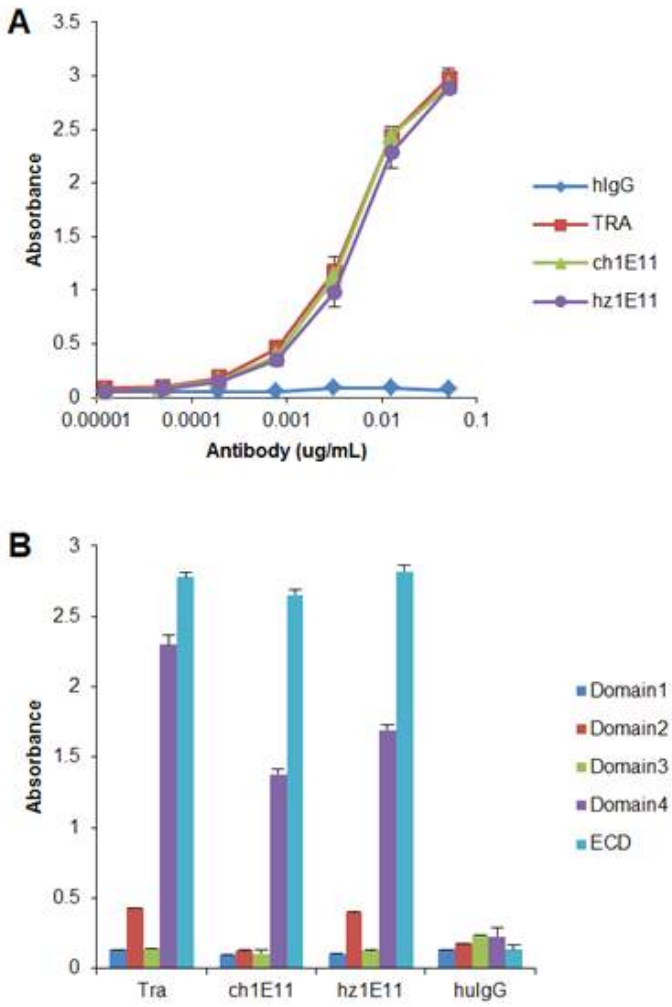
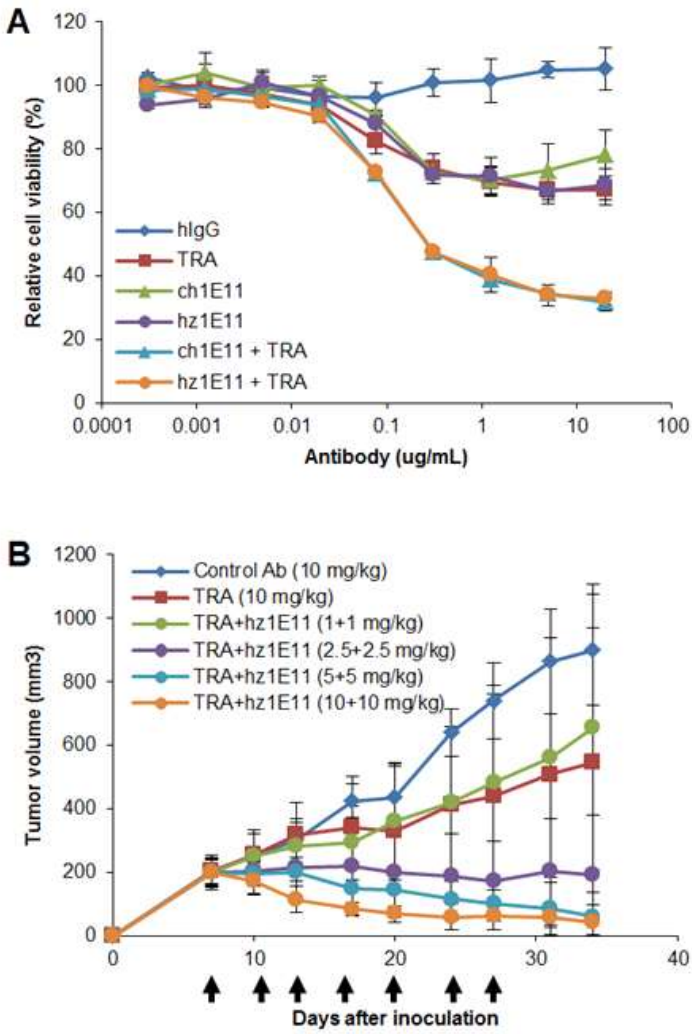

A
Absorbance
Antibody (ug/mL)
0.00001
0.0001
0.001
0.01
0.1
hIgG
TRA
ch1E11
hz1E11
B
Absorbance
Tra
ch1E11
hz1E11
huIgG
Domain1
Domain2
Domain3
Domain4
ECD

Figure 21. Humanized 1E11 shows synergistic antitumor activity in combination with trastuzumab.

A, NCI-N87 cells were treated with antibodies for 4 days in the complete growth media. Data points of cell viability assay are mean ± SD (n = 3) and the 100% point was defined as the absorbance of antibody untreated well. **B**, Mice bearing NCI-N87 xenograft tumors were treated with indicated dose of control antibody, trastuzumab, hz1E11, or trastuzumab + hz1E11. Palivizumab was used as the isotype control antibody. Administration days are indicated by arrows. Tumor volume (mm^3) was expressed as mean ± SD (n = 6 mice/group).


A
Relative cell viability (%)
120
100
80
60
40
20
0
hIgG
TRA
ch1E11
hz1E11
ch1E11 + TRA
hz1E11 + TRA
0.0001
0.001
0.01
0.1
1
10
100
Antibody (ug/mL)
B
Tumor volume (mm3)
1200
1000
800
600
400
200
0
Control Ab (10 mg/kg)
TRA (10 mg/kg)
TRA+hz1E11 (1+1 mg/kg)
TRA+hz1E11 (2.5+2.5 mg/kg)
TRA+hz1E11 (5+5 mg/kg)
TRA+hz1E11 (10+10 mg/kg)
0
10
20
30
40
Days after inoculation

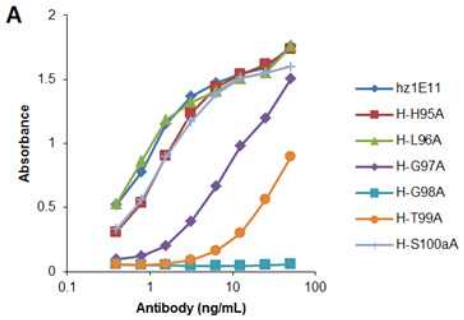
like the parental ch1E11 (Fig. 20B). Trastuzumab is also known to bind to sub-domain IV (Cho et al., 2003). The *in vitro* antiproliferative activities of hz1E11 as single agent and in combination with trastuzumab are also equivalent to those of ch1E11 (Fig. 21A). The combination of hz1E11 with trastuzumab showed dose-dependent antitumor activity in NCI-N87 xenograft mouse model (Fig. 21B). The result also shows that antibody combination treatment at 1 mg/kg each of hz1E11 and trastuzumab resulted in similar antitumor activity with trastuzumab single treatment at 10 mg/kg, and established tumors were started to regress from at 5 mg/kg combination. These results show that hz1E11 has equivalent affinity and biological activity to ch1E11, and hz1E11 enhance the antitumor activity of trastuzumab when used in combination.

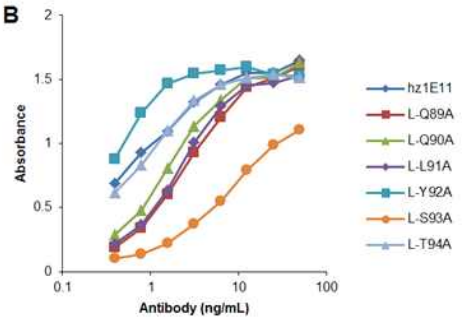
Alanine scanning of CDR-H3 and CDR-L3

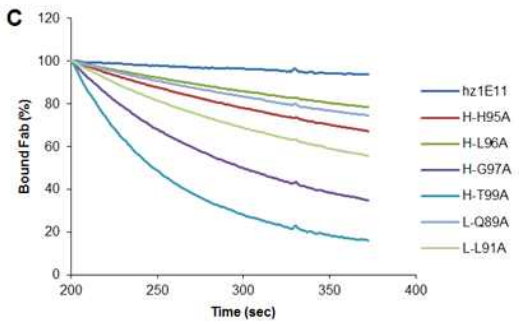
To identify the critical residues for antigen-antibody interaction, we conducted alanine scanning mutagenesis in CDR3 regions of heavy and light chain (CDR-H3 and CDR-L3). The effects of the mutations were assessed by analyzing binding activity of purified Fab proteins by indirect ELISA. In CDR-H3, alanine substitution at position $Gly98_H$ abolished the binding of the Fab to HER2, and $G97_HA$ and $T99_HA$ mutants showed reduced binding activity (Fig. 22A). In CDR-L3, alanine substitution at position $Ser93_L$ resulted in reduced binding and $Y92_LA$ mutants showed enhanced binding activity (Fig. 22B). The k_{off}

Figure 22. Three residues in heavy chain CDR3 are hot-spot for antigen-antibody binding.

The binding activities of alanine scanning mutants of heavy chain (A) and light chain (B) were analyzed by ELISA. C, Quantitative dissociation kinetics of indicated mutants were analyzed. HER2-ECD protein was immobilized on a CM5 sensor chip followed by exposure to indicated mutant Fab antibodies. 100% point was defined as the response unit (RU) at 200 seconds from experiment start. Koff values were calculated using the BIAevaluation software.







D

Clone	Koff (1/s)
hz1E11	3.59e-4
H-L96A	1.44e-3
L-Q89A	1.74e-3
H-H95A	2.33e-3
L-L91A	3.44e-3
H-G97A	6.25e-3
H-T99A	1.18e-2

values of alanine scanning mutants were analyzed using SPR against immobilized HER2-ECD protein. We confirmed that heavy chain G98A mutant completely lost binding activities, and the neighboring $T99_HA$ and $G97_HA$ mutants resulted in 32-fold and 17-fold increased k_{off} values, respectively (Fig. 22C and 22D). These results indicate that heavy chain $Gly98_H$ is functionally critical and its adjacent residues are also functionally important for antigen-antibody interaction.

Affinity maturation of hz1E11

Affinity maturation of hz1E11 was conducted by the construction of heavy or light chain CDR3 randomized library, followed by the equilibrium selection of phage antibody library. For light chain, glutamine residues at position 89_L and 90_L were excluded because they are commonly found in 9 amino acid length CDR-L3 at 59% and 73% frequencies, respectively (Martin, 1996). Positions 91_L - 94_L were randomized using NNK degenerated codon (L-NNK). For heavy chain, positions 95_H-$100a_H$ were randomized using XXS codon (H-XXS), which were designed so that at the first and second positions of the diversified codon the wild type base occurs with 70% chance and each of the other three bases occur at 10% frequency, and the third letters of the codons were synthesized using an equimolar mixture of G and C (Fig. 23A). High affinity binders were selected from high-stringency or low-stringency panning (Fig. 23B).

Figure 23. Library design and panning strategy

A, For light chain, positions 91_L - 94_L were randomized using NNK degenerated codon (L-NNK). For heavy chain, positions 95_H-$100a_H$ were randomized using XXS codon (H-XXS) or twin NNS random codon scanning of CDR-H3 (H-2AA). **B**, High affinity binders were selected from high-stringency or low-stringency panning.

A

L-NNK library

```
  Q   Q   L   Y   S   T   P   W   T
CAG CAG CTT TAT AGC ACC CCC TGG ACC
CAG CAG NNK NNK NNK NNK CCC TGG ACC
```

H-XXS library

```
  H   L   G   G   T   A   S   F   D   Y
CAC CTG GGT GGG ACC GCC TCC TTC GAC TAC
XXS XXS XXS XXS XXS XXS XXS TTC GAC TAC
```

H-2AA library

```
  H   L   G   G   T   A   S   F   D   Y
CAC CTG GGT GGG ACC GCC TCC TTC GAC TAC
NNK NNK GGT GGG ACC GCC TCC TTC GAC TAC
CAC NNK NNK GGG ACC GCC TCC TTC GAC TAC
CAC CTG NNK NNK ACC GCC TCC TTC GAC TAC
.   .   .
CAC CTG GGT GGG ACC NNK NNK TTC GAC TAC
```

B

	Panning-1 (high-stringency)	Panning-2 (low-stringency)
1st round	0.1 nM	100 nM
2nd round	10 pM	1 nM
3rd round	1 pM	0.1 nM
4th round	0.1 pM	10 pM

Compared to the low-stringency panning in which 100% of the second, third and fourth round output clones screened were ELISA positive, for the high-stringency panning the fourth round output yielded no binder at all, and only one third of the third round output clones were ELISA positive (Table 1). The selected clones from the CDR-L3 library included many sequences that had all four randomized CDR-L3 positions mutated, unlike CDR-H3 libraries from which most of the selected clones retained the wild type sequence in positions 97_H-100_H (see below). Different panning strategies yielded different sequence enrichment pattern: for example, the light chain variant clone 1A12 (Q_{89L}QNAYAPWT$_{97L}$) was isolated from the high-stringency panning but not from the low-stringency panning, and the heavy chain variant clone 1B12 (N_{95H}YGGTASFDY$_{102H}$) was selected only from the high-stringency panning. Interestingly, 59% of unique antibodies in 4^{th} output with low-stringency panning of L-NNK library had an alanine at position 92_L, in line with alanine scanning analysis (Fig. 24A).

Almost all clones that were isolated from the H-XXS library had the same sequence at 97_H - 100_H as the parental clone, and showed a clear enrichment of Asn-Tyr sequence at 95_H - 96_H. To further assess the contribution of CDR-H3 residues to antigen-antibody binding, we constructed additional CDR-H3 library with twin NNS random codon scanning of CDR-H3 (H-2AA). In low-stringency panning, mutants at positions 95_H, 96_H, 99_H, 100_H, and $100a_H$ were selected in first round panning, but only mutants at positions 95_H and 96_H were

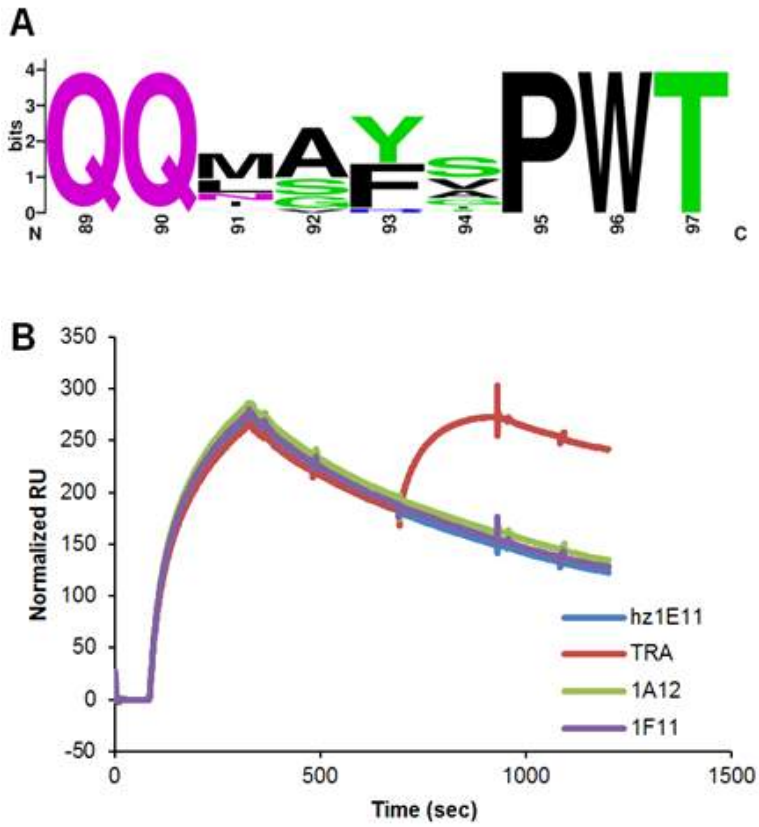
Table 1. Panning results of CDR-L3 and CDR-H3 library

		ELISA positive ratio				Diversity 1)	
Library	Strategy	1st output	2nd output	3rd output	4th output	Output	CDR-L3 OR - H3 (colony/tested colony)
L-NNK	Panning-1	73%	80%	33%	0%	3rd output	QQNAYAPWT(15/34) QQLGFIPWT(10/34) QQNAFSPWT(2/34)
	Panning-2	80%	100%	100%	100%	4th output	QQTAFSPWT(4/55) QQIAYVPWT(3/55) QQMSYGPWT(3/55) QQMSYVPWT(3/55)
H-XXS	Panning-1	73%	50%	20%	3%	2nd output	NYGGTASFDY (8/9) HFGGTASFDY(1/9)
	Panning-2	87%	100%	100%	100%	4th output	NFGGTASFDY (3/5) HFGGTASFDY(1/5) HLGGTASFDY (1/5)
H-2AA	Panning-1	73%	30%	33%	10%	2nd output	NYGGTASFDY (5/7) NLGGTASFDY (2/7)
	Panning-2	73%	97%	100%	100%	4th output	NFGGTASFDY (6/10) NYGGTASFDY (2/10) HWGGTASFDY(1/10) HYGGTASFDY(1/10)

1) For diversity of L-NNK library with panning-2, only 4 enriched clones are presented. Substituted amino acids are underlined.

Figure 24. Sequence and binding properties of affinity-matured clones

A, Sequence logo were generated using WebLogo program about unique clones in 4th output of CDR-L3 library with low-stringency panning (n = 39). **B**, The hz1E11 was immobilized on a CM5 sensor chip followed by sequential exposure to HER2-ECD-His and indicated antibodies.


A
bits
4
3
2
1
0
N
89
90
91
92
93
94
95
96
97
C
B
Normalized RU
350
300
250
200
150
100
50
0
-50
0
500
1000
1500
Time (sec)
hz1E11
TRA
1A12
1F11

enriched in 4th round output (Table 1). We could not detect the mutants at positions $Gly97_H$ and $Gly98_H$ in any panning strategies and outputs.

Affinity and binding activity of selected clones

To assess the combination effect of the affinity-matured heavy and light variants, IgG antibodies with combinations of affinity-matured heavy and light chains were produced and their k_{off} values were determine using SPR analysis (Table 2). Among two light chain variants and two heavy chain variants, 1A12 derived from L-NNK showed the highest improvement (16-fold). The combination of the light chain of 1A12 with the optimized heavy chain variants improved k_{off} value such that the combination of 1A12 light chain and 1B12 heavy chain resulted in a 24-fold improvement in k_{off} over the parental hz1E11. Because the improvement by this combination was not significantly large, clones 1A12 and 1F11 (light chain variants) were chosen for further characterization to minimize the sequence difference of the affinity-matured antibodies from the parental clone.

To determine whether affinity-matured hz1E11 clones bind to the same epitope as hz1E11, the binding of 1A12 and 1F11 to the HER2-ECD that was pre-bound to hz1E11 was analyzed by SPR. Both clones were unable to bind to the monomeric HER2-ECD-His protein captured by immobilized 1E11 whereas trastuzumab could

Table 2. Koff measurement of selected clones

CLONE	SEQUENCE		Koff (1/s)
	CDR-L3	CDR-H3	
hz1E11	QQLYSTPWT	HLGGTASFDY	4.48E-4
L-1A12	QQNAYAPWT	HLGGTASFDY	2.80E-5
L-1F11	QQTAFSPWT	HLGGTASFDY	5.89E-5
H-1B12	QQLYSTPWT	NYGGTASFDY	7.40E-5
H-2A7	QQLYSTPWT	NFGGTASFDY	5.20E-5
L-1A12 + H-1B12	QQNAYAPWT	NYGGTASFDY	1.87E-5
L-1F11 + H-1B12	QQTAFSPWT	NYGGTASFDY	3.45E-5
L-1A12 + H-2A7	QQNAYAPWT	NFGGTASFDY	2.59E-5
L-1A12 + H-2A7	QQTAFSPWT	NFGGTASFDY	4.50E-5

- Substituted amino acids are underlined.

(Fig. 24B). This data indicate that epitopes of 1A12 and 1F11 does not change by affinity maturation process.

Efficacy of affinity matured hz1E11 clones

Both 1A12 and 1F11 antibodies showed slightly increased antiproliferative activities as a single agent compared to hz1E11 on NCI-N87 and OE-19 gastric cancer cell lines that overexpress HER2 (Fig. 25A and 25B), whereas in combination with trastuzumab, their antiproliferative activities was superior to that trastuzumab alone and equivalent to hz1E11 plus trastuzumab. The antiproliferative activity of 1A12 and 1F11 was confirmed *in vivo* in NCI-N87 and OE-19 xenograft models. They showed superior antitumor activity in combination with trastuzumab to that of each agent alone in both xenograft models (Fig. 26A and 26B).

Figure 25. Affinity-matured hz1E11 clones shows antiproliferative activity in HER2-overexpressing gastric cancer models

A, NCI-N87 cells and **B**, OE-19 cells were treated with antibodies for 4 days in the complete growth media. Data points of cell viability assay are mean ± SD (n = 3) and the 100% point was defined as the absorbance of antibody untreated well.

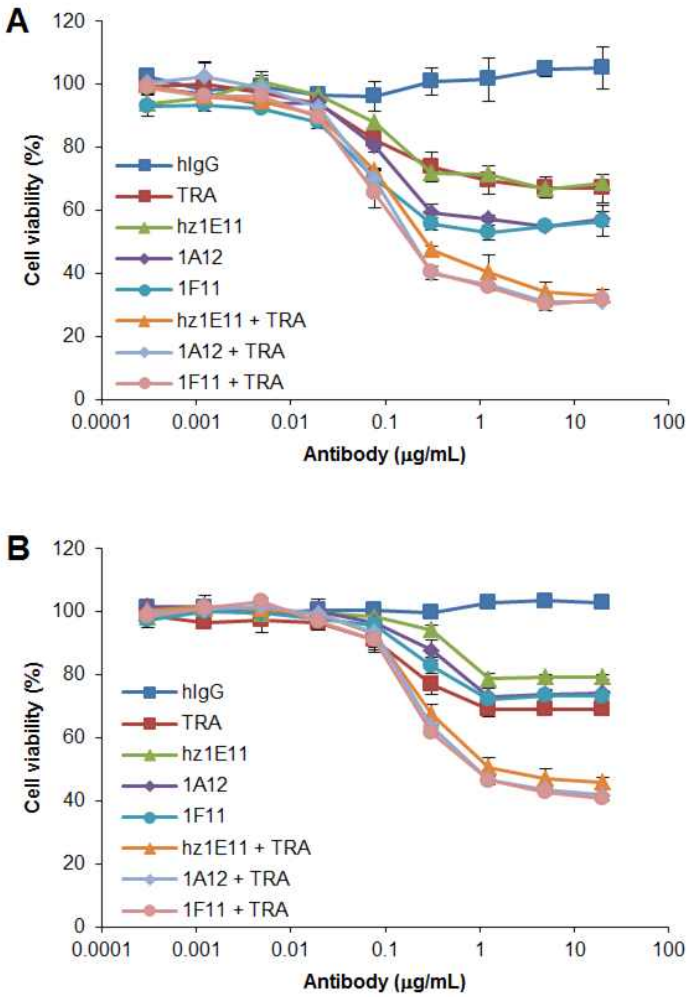
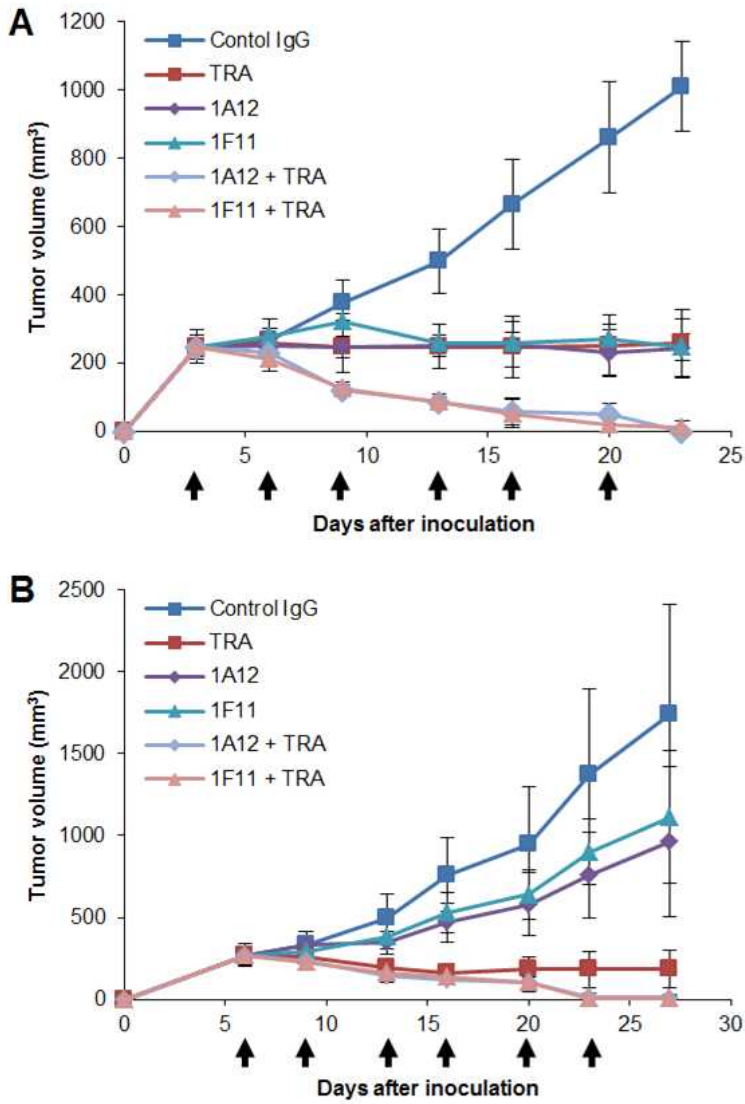

A
120
100
80
60
40
20
0
Cell viability (%)
hIgG
TRA
hz1E11
1A12
1F11
hz1E11 + TRA
1A12 + TRA
1F11 + TRA
0.0001
0.001
0.01
0.1
1
10
100
Antibody (μg/mL)
B
120
100
80
60
40
20
0
Cell viability (%)
hIgG
TRA
hz1E11
1A12
1F11
hz1E11 + TRA
1A12 + TRA
1F11 + TRA
0.0001
0.001
0.01
0.1
1
10
100
Antibody (μg/mL)

Figure 26. Antitumor activity of affinity-matured hz1E11 clones in xenograft models

A, NCI-N87 (n = 5 mice/group) and **B**, OE-19 (n = 6 mice/group) cells were inoculated into mice and antibody treatments were started when tumor volumes reached approximately 200 mm^3. Mice received a dose of 20 mg/kg for single agent treatment and 10 mg/kg of each antibody for combination treatment. Administration days are indicated by arrows. Tumor volume (mm^3) was expressed as the mean ± SD.


A
Contol IgG
TRA
1A12
1F11
1A12 + TRA
1F11 + TRA
Tumor volume (mm³)
Days after inoculation
B
Control IgG
TRA
1A12
1F11
1A12 + TRA
1F11 + TRA
Tumor volume (mm³)
Days after inoculation

Discussion

Despite encouraging clinical results obtained with the HER2 targeting antibody trastuzumab in the treatment of gastric cancer, it is still necessary to develop the more potent HER2 targeted therapies (Bang et al., 2010). Combination of non-competing antibodies targeting receptors such as HER2, EGFR, and VEGFR3 can increase antitumor activity in preclinical models (Kamat et al., 2008; Pedersen et al., 2010; Tvorogov et al, 2010; and Zhang et al., 2010). Pertuzumab, another HER2-targeting antibody, is approved for use in combination with trastuzumab in the treatment of metastatic breast cancer (Baselga et al., 2012). In the previous study, we developed a novel HER2 targeting antibody, 1E11, which shows significant antitumor activity as a single agent and synergistic effect in combination with trastuzumab in *in vitro* and *in vivo* models (in press). The antitumor activity of 1E11 and trastuzumab combination is superior not only to trastuzumab single treatment but also to the combination of pertuzumab and trastuzumab. In this report, the humanization and subsequent affinity maturation of the mouse hybridoma-derived 1E11 monoclonal antibody is described.

Initial approaches to reduce potential immunogenicity of nonhuman variable regions by CDR-grafting into a human framework significantly decreased the immunogenicity of therapeutic antibodies (Jones et al., 1986 and Hwang and Foote, 2005). The

superhumanization using human germline frameworks as template has been proposed as a superior humanization methodology to avoid putative effector T-cell epitopes derived by somatic hypermuations of human framework (Tan et al., 2002; Hwang et al., 2005; Pelat et al., 2008). It has been suggested that antibodies encoded by germline gene segments are structurally flexible and able to accommodate binding to many different antigens (James et al., 2003; Wong et al., 2011; Yin et al., 2003). The CDRs of murine antibody 1E11 as well as one residue in the Vernier zone of V_H ($Ala49_H$) were grafted on to the human germline variable and joining genes with highest homology to the parental antibody (Fig. 19). The resulting humanized antibody, hz1E11, shows almost identical affinity (Fig. 20) and biological activity to the parental antibody (Fig. 21).

Selection of residues for randomization is a critical step in targeted randomization approach of affinity maturation because of the practical limitations of antibody library size and the selection technologies. Alanine scanning and *in silico* analysis of the antibody sequence, especially in CDRs, are useful for selecting the randomization target residues and facilitate the affinity maturation process. In alanine scanning analysis of CDR-H3 of hz1E11, $G98_HA$ mutant completely lost binding activity, and $G97_HA$ and $T99_HA$ mutants showed reduced binding activity (Fig. 22A). Direct alteration of an essential paratope residue usually results in the total loss of binding ability of the antibody (Cho et al., 2003; James et al., 2003; Kelley and O'Connell; 1993); therefore, a more conservative approach to CDR-H3

randomization was employed, using either a "hand-mixed" oligonucleotide that is biased toward the parental sequence, or the twin NNS random codon scanning of CDR-H3. As expected from the alanine scanning analysis, almost all clones that were isolated from the CDR-H3 libraries had the sequence 97_H - $100a_H$ same as the parental clone ($G_{97H}GTAS_{100aH}$).

The alanine scanning results suggest that all CDR-L3 residues are dispensable, although the mutation of $Ser93_L$ to alanine seems to lower the affinity (Fig. 22B). Therefore, four positions of CDR-L3 (91_L - 94_L) were fully randomized using NNK degenerate codon. The sequence analysis of the selected clones confirms the results from the alanine scanning analysis; the majority of the selected clones from the CDR-L3 library had all four randomized CDR-L3 positions changed from the parental residue, contrary to the CDR-H3 optimization results in which most of the selected clones from the libraries have the same sequence as the parental hz1E11 in the middle part of the CDR (Table 1). It is also noted that the clones with most affinity improvement came from the CDR-L3 library. It is probable that the CDR-H3 of hz1E11 is already close to optimal and most mutations, especially in the region 97_H - 100_H, are deleterious to the binding affinity, while the CDR-L3 sequence is not as optimal and the mutation in this region can result in significant improvement in the affinity. The L-NNK library panning outputs were enriched with clones with a hydrophobic amino acid at the position 91_L, a small amino acid at 92_L, and an aromatic amino acid at 93_L; this

pattern is some what different from the parental CDR-L3 sequence of Q_{89L}QLYSTPWT$_{97L}$ and again suggests that the CDR-L3 sequence of the parental 1E11 antibody was sub-optimal and had some opportunity for affinity improvement.

The clones with most improvement in affinity, 1A12 and 1F11, came from the high-stringency panning of the CDR-L3 NNK library, although 1F11 was also found from the low-stringency panning of the same library (Table 2). Compared to the low-stringency panning in which 100% of the second, third and fourth round output clones screened were ELISA positive, for the high-stringency panning the fourth round output yielded no binder at all, and only one third of the third round output clones were ELISA positive (Table 1). It is likely that the extremely antigen-limiting condition favored clones with higher affinity, however at the same time the low level of non-specific binding of phage particles became more evident, and even dominated the panning output. In agreement with this argument, sequence analysis of the ELISA positive clones showed that the high-stringency panning resulted in the convergence of the output clones to a small number of sequences, while the sequences were more divergent for the output clones from the low-stringency panning. For the CDR-H3 libraries, the overall pattern of the panning output titer and the ratio of ELISA positive clones were similar to that of the CDR-L3 libraries; i.e. higher panning stringency had a negative effect on both parameters. Not surprisingly, the highly enriched clone (1B12) with CDR-H3 sequence N_{95H}YGGTAS$_{100aH}$ had

k_{off} value that was more than 5 times slower than that of the parental antibody. The combination of the V_H of 1B12 and V_L of 1A12 further improved k_{off} and K_D slightly.

The humanized, affinity matured anti-HER2 clones showed inhibitory activity on the growth of HER2-positive gastric cancer cell lines *in vitro* and *in vivo* xenograft models. The inhibitory activity does not reflect the improvement in the binding affinity, however, and their maximal growth inhibition and IC_{50} values are comparable to those of the parental hz1E11, possibly because the hz1E11 already had sufficiently high inhibitory activity on HER2-overexressing cancer cell lines. Of course, this does not necessarily mean that they would have similar clinical efficacies, and the effect of the affinity improvement of hz1E11 on the growth of HER2-positive human tumors needs to be evaluated in future development research.

To summarize, an anti-HER2 murine antibody 1E11 was humanized and affinity matured through extensive library construction, panning, and screening efforts. High-stringency, antigen-limiting selection conditions facilitated the isolation of affinity matured clones, among which the best ones were CDR-L3 variants. Different CDR diversification strategies produced different sequence enrichment patterns, with varying degrees of success in affinity improvement. On the other hand, the stringency of panning had similar effects on the output pattern for all the libraries. It is anticipated that these results can be utilized to improve the experimental design of future affinity

maturation efforts and generate highly optimized antibody clones for therapeutic and other applications.

CONCLUSION

Gastric cancer is one of the leading types of cancer worldwide. Although the trend in death rates for gastric cancer is decreasing, prognosis is poor and few therapeutic options are available, particularly in advanced stages. HER2 is a type 1 transmembrane receptor tyrosine kinase (RTK) and member of the epidermal growth factor receptor (EGFR/ErbB) family and it induces the transduction of signaling *via* two major pathways, which are the Ras/Raf/MEK/ERK and the PI3K/AKT/mTOR pathways. And these pathways promote cell proliferation, inhibition of apoptosis, angiogenesis, and invasion which leading to tumor growth and progression. The frequency of HER2-positive gastric cancer is 22.1% based on ToGA trial. Trastuzumab, trade name HERCEPTIN, is HER2-targeting therapeutic antibody and approved for the treatment of HER2-overexpressing metastatic gastric cancer based on ToGA trial. Recent evidence suggests that particular combinations of noncompetitive antibodies targeting the same receptor increase antitumor activity *in vitro* and *in vivo*.

In this study, I demonstrate that 1E11 shows significant antitumor activity as single agent in *in vitro* and *in vivo* HER2-positive gastric cancer models. Antitumor activity of 1E11 is increased in a highly synergistic manner in combination with trastuzumab. The two

antibodies bind to sub-domain IV of the receptor, but have non-overlapping epitopes, allowing them to simultaneously bind HER2. Treatment with 1E11 alone induced apoptosis in HER2-positive cancer cells, and this effect was enhanced by combination treatment with trastuzumab. Combination treatment with 1E11 and trastuzumab reduced the levels of total HER2 protein and those of aberrant HER2 signaling molecules including phosphorylated HER3 and EGFR. 1E11 was further optimized to reduce the immunogenicity and to increase the affinity through humanization and affinity maturation. I selected 1A12 clone which has 4 amino acid mutations in CDR-L3 and showed its improved affinity against HER2 protein more than 10-fold. I confirm that antitumor activity of 1A12 is correlated with HER2 levels in more than 15 gastric and breast cancer cell panel. The antibody-dependent cellular cytotoxicity (ADCC) activity of 1A12 is comparable to that of trastuzumab and pertuzumab. In combination setting with trastuzumab, 1E11 completely inhibits tumor growth in OE-19 xenograft model, while pertuzumab causes partial inhibition of tumor growth. Taken together, I propose that combination treatment of 1A12 and trastuzumab could be a novel potent therapeutic strategy for the treatment of patients with HER2-overexpressing gastric and breast cancer.

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CHAPTER I

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요약 (국문초록)

본 연구에서는 HER2를 표적하며 HER2 과발현 위암에서 단독으로 항암 효능을 보일뿐 아니라 trastuzumab과 병용처리 시 동반 상승효과를 보이는 신규 항체의 효능, 작용기전, 항체 최적화에 대한 연구가 수행되었다.

위암은 세계적으로 많은 발생빈도를 보이는 암이다. 위암에 의한 사망률이 점차 낮아지는 추세이긴 하지만, 예후가 좋지 않고 적절한 치료 방법이 없는 상태이다. HER2는 세포막 수용체 타이로신 인산화효소 (receptor tyrosine kinase, RTK)로 EGFR/ErbB (epidermal growth factor receptor) family에 속하며, HER2 이외에도 EGFR, HER3, HER4 단백질들이 속해있다. ErbB 단백질들은 리간드와 결합하는 세포외 도메인, 세포투과 도메인, 세포내 도메인으로 구성되어 있다. HER2를 제외한 ErbB 단백질들은 접힌 상태로 되어 이합체화를 유도하는 서브도메인 II (sub-domain II)가 숨겨져 있는 상태로 존재한다. HER2의 주요 신호전달계는 Ras/Raf/MEK/ERK와 PI3K/AKT/mTOR이며, HER2는 이들 신호전달계를 통하여 암의 성장 및 진행과 관련된 세포 증식, 세포 사멸 억제, 혈관생성, 세포이동 등을 유도한다.

HER2 단백질의 과발현은 위암 환자의 22.1%에서 관찰됨이 ToGA 임상시험을 통하여 확인되었다. HER2 과발현과 위암의 예후와의 상관관계에 대해서는 모순되는 보고가 있으나, 많은 연구들이 상관관계가 있음을 보고하고 있다. Trastuzumab (제품명

HERCEPTIN)은 HER2 를 표적하는 항체로 HER2 과발현 유방암에 대한 치료제이며 최근에는 HER2 과발현 전이성 위암에 대한 치료제로도 승인받았다. ToGA 임상시험에 따르면 trastuzumab 처리와 화학요법을 병용시행 시 평균 전체 생존율이 13.8 개월로 화학요법만 시행한 경우인 11.1 개월 보다 향상되었다. Trastuzumab 이 임상적 효능을 보이고는 있지만 보다 향상된 효능을 보이는 치료법의 개발이 필요하다. 최근 동일 단백질을 표적하는 항체들의 병용처리에 의한 항체치료 효능의 향상이 보고되고 있다. 대표적인 예인 pertuzumab 은 HER2 를 표적하는 항체로, trastuzumab 이 서브도메인 IV 에 결합하는 반면 pertuzumab 은 서브도메인 II 에 결합한다. Pertuzumab 은 단독으로는 항암 효능이 미비하지만 trastuzumab 과 병용처리하면 각 항체를 단독 처리한 경우보다 향상된 효능을 보인다. 이와 같은 병용처리의 예는 EGFR 과 VEGFR3 를 표적하는 항체들에 대해서도 보고되었다.

본 연구에서는 HER2 를 표적하는 신규 단클론항체인 1E11 의 효능과 작용기전을 HER2 과발현 위암과 유방암을 대상으로 수행되었다. 1E11 은 HER2 과발현 위암의 in vitro 및 in vivo 모델에서 단독으로도 의미 있는 항암 효능을 보일뿐만 아니라 trastuzumab 과 병용투여 시에 동반 상승효과를 보였다. 두 항체 모두 서브도메인 IV 에 결합하지만 서로 epitope 를 공유하지 않기 때문에 HER2 에 동시에 결합하였다. 1E11 항체는 단독으로 HER2 과발현 암의 세포사멸을 유도하였으며, 이런 활성은 trastuzumab 과 병용처리 시에 상승하였다. 1E11 과 trastuzumab 을 동시처리 시에 전체 HER2 단백질이 감소하였으며 인산화된 형태의 EGFR 과 HER3 의 양도 감소되었다. 1E11 는 생쥐 유래 항체이기 때문에 인간에 투여 시 가변영역에 대한 면역반응이 유도될 수 있다. 이를 극복하기 위해서 인간 germline 항체를 골격으로

한 항체에 상보성결정지역을 이식하는 방법으로 인간화 항체를 개발하였다. 이렇게 개발된 인간화 항체인 hz1E11 은 1E11 과 동등한 친화도와 생물학적 활성을 보였다. 또한 hz1E11 의 친화도 향상을 위해서 중쇄사슬 및 경쇄사슬의 상보성결정지역 3 에 다양성을 부여하고 파지디스플레이 (phage display) 기술을 이용하여 친화도가 향상된 항체를 선별하였다. 선별된 1A12 항체는 경쇄사슬의 상보성결정지역 3 에 4 개의 아미노산이 변형된 항체로 10 배 이상 HER2 단백질에 대한 친화도가 향상된 항체이다. 그리고 1A12 의 항암 활성이 HER2 의 발현 정도와 관련이 있음을 15 개 이상의 위암 및 유방암 세포를 대상으로 확인하였다. 또한 1A12 항체가 trastuzumab 이나 pertuzumab 과 동등한 항체의존 세포독성을 보임을 확인하였다. Trastuzumab 과 병용처리 시에 1A12 는 OE-19 세포를 이용한 이종이식 모델에서 암의 성장을 완전히 억제한 반면 pertuzumab 은 암의 성장을 다소 억제하는데 그쳤다.

결과적으로 1E11 은 trastuzumab 과 병용처리 시에 HER2 과발현 위암의 억제에 동반 상승효과를 보였다. 1E11 의 항암효능은 세포사멸 유도 및 HER2 세포신호전달계 억제를 통하여 나타난다. 따라서 이들 항체의 병용처리는 HER2 과발현 위암 환자에 대한 새로운 치료제로서 높은 가능성을 갖고 있다.

주요어: HER2, 위암, 항체, 병용, 상승효과

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