Molecular Cancer Therapeutics

Construction and Characterization of Novel, Completely Human Serine Protease Therapeutics Targeting Her2/neu

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Abstract

Immunotoxins containing bacterial or plant toxins have shown promise in cancer-targeted therapy, but their long-term clinical use may be hampered by vascular leak syndrome and immunogenicity of the toxin. We incorporated human granzyme B (GrB) as an effector and generated completely human chimeric fusion proteins containing the humanized anti-Her2/neu single-chain antibody 4D5 (designated GrB/4D5). Introduction of a pH-sensitive fusogenic peptide (designated GrB/4D5/26) resulted in comparatively greater specific cytotoxicity although both constructs showed similar affinity to Her2/neu–positive tumor cells. Compared with GrB/4D5, GrB/4D5/26 showed enhanced and long-lasting cellular uptake and improved delivery of GrB to the cytosol of target cells. Treatment with nanomolar concentrations of GrB/4D5/26 resulted in specific cytotoxicity, induction of apoptosis, and efficient downregulation of PI3K/Akt and Ras/ERK pathways. The endogenous presence of the GrB proteinase inhibitor 9 did not impact the response of cells to the fusion construct. Surprisingly, tumor cells resistant to lapatinib or Herceptin, and cells expressing MDR-1 resistant to chemotherapeutic agents showed no cross-resistance to the GrB-based fusion proteins. Administration (intravenous, tail vein) of GrB/4D5/26 to mice bearing BT474 M1 breast tumors resulted in significant tumor suppression. In addition, tumor tissue excised from GrB/4D5/26–treated mice showed excellent delivery of GrB to tumors and a dramatic induction of apoptosis compared with saline treatment. This study clearly showed that the completely human, functionalized GrB construct can effectively target Her2/neu–expressing cells and displays impressive *in vitro* and *in vivo* activity. This construct should be evaluated further for clinical use. *Mol Cancer Ther; 12(6); 979–91. ©2013 AACR.*

Introduction

Bacterial and plant toxin-based immunotoxins have shown remarkable potency and specificity, but a number of obstacles limit their clinical application (1, 2). The toxin component of these fusion proteins can elicit vascular damage leading to loss of vascular integrity (vascular leak syndrome, VLS; refs. 3, 4). Immune responses to the toxins in patients also result in rapid clearance of subsequent courses of therapy (5, 6). Toxin immunogenicity is being addressed by engineering B-cell epitopes on the structure (7, 8), but these molecules may be difficult to humanize completely (9).

A new class of immunotoxins have recently been developed containing cytotoxic human proteins (10, 11). Granzyme B (GrB) is a well-known serine protease generated by cytotoxic lymphocytes to induce apoptotic cell death in target cells (12, 13). Our group first showed that various fusion constructs targeting tumor cells and tumor endothelium and containing GrB have impressive proapoptotic and cytotoxic activity (14–18). Several other groups have since confirmed these findings using other GrB-containing constructs (19, 20). Because endogenous GrB is present in plasma in both normal and pathologic states, it is unlikely that this molecule would engender an immune response.

Dalken and colleagues have described a GrB/FRP5 fusion construct targeting Her2/neu, which displayed selective and rapid tumor cell killing *in vitro* (21). However, studies have shown that the fusion construct required the presence of the endosome-disrupting agent chloroquine for biologic activity and suggested that an endosomal release process may be necessary for Her2/neu–targeted agents. Studies by Wang and colleagues suggested that incorporation of a furin-sensitive linker into GrB-based fusion constructs may promote effective

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cytoplasmic delivery of an active GrB fragment into target cells (22). However, the recombinant molecule seemed to be stable only when generated *in situ* by protein-expressing transfected cells.

We previously examined a series of anti-Her2/neu single-chain antibodies (scFv) fused to the recombinant gelonin (rGel) toxin, and clearly showed that scFvs with intermediate affinity (K_d 10^{-11} mol/L) as opposed to high affinity (K_d 10^{-12} mol/L) were optimal carriers of protein toxins (23, 24). Therefore, we used an intermediate-affinity, humanized anti-Her2/neu scFv-designated 4D5 for the construction of our GrB-containing fusion constructs. In this study, we provided data on the cytotoxicity of Her2/neu–targeted GrB fusions against a panel of human tumor cell lines and explored the mechanism of *in vitro* activity of these fusion constructs. Finally, we showed the *in vivo* antitumor efficacy of the functionalized GrB chimeric protein against a human breast xenograft model.

Materials and Methods

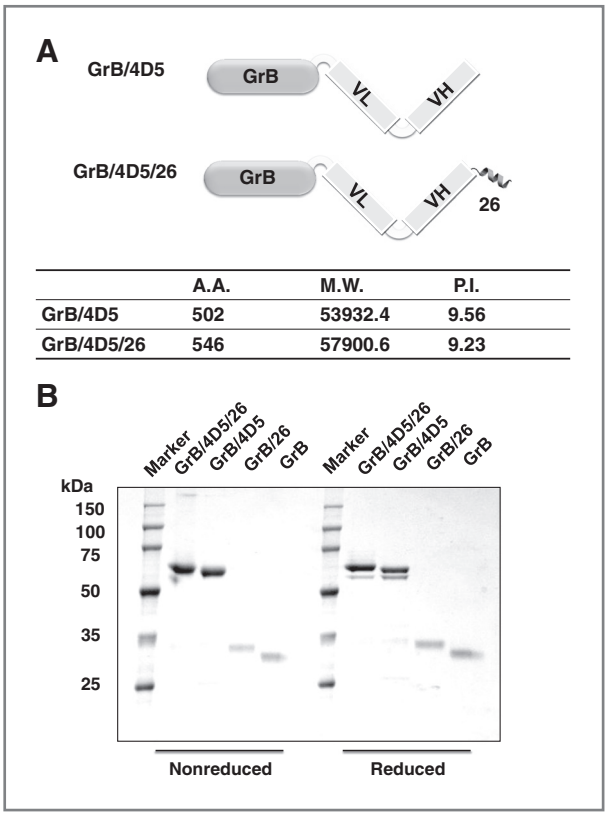
Cell lines

The cell lines BT474 M1, NCI-N87, Calu3, MDA MB435, and Me180 were all obtained from American Type Culture Collection (ATCC). The human breast cancer cell lines MDA MB453 and eB-1 were generously supplied by Drs. Zhen Fan and Dihua Yu (MD Anderson Cancer Center, Houston, TX). The BT474 M1 Herceptin- and lapatinib-resistant cells were derived from BT474 M1 cells after a 12-month selection in the continuous presence of 1 μmol/L Herceptin or 1.5 μmol/L lapatinib. BT474 M1 MDR-1 cells were generated by the transfection of plasmid pHaMDR1 to parental BT474 M1 cells. The HEK 293T cell line was supplied by Dr. Bryant G. Darnay (MD Anderson Cancer Center). All cell lines were maintained in Dulbecco's Modified Eagle Medium or RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, and 1 mmol/L antibiotics.

Cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpFℓSTR Identifiler Kit according to the manufacturer's instructions (Applied Biosystems). The STR profiles were compared with known ATCC fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/; Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526) and to the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique.

Plasmid construction

The GrB/4D5/26, GrB/4D5, GrB/26, and GrB DNA constructs were generated by an overlapping PCR method. Illustrations of the constructs are shown in Fig. 1A. We designed a universal 218 linker (GSTSGSGKPGSGEGSTKG) incorporated between the individual components of GrB, 4D5, or peptide 26. Peptide 26 (AALEALAEALEALAEALEALAEAAAA) was generated from the 29-residue amphipathic peptide without the 3 C-terminal amino acids, which are responsible for dimerization (25). All construct genes were cloned into the mammalian cell expression vector pSecTag (Life Technologies).



	A.A.	M.W.	P.I.
GrB/4D5	502	53932.4	9.56
GrB/4D5/26	546	57900.6	9.23

Figure 1. Construction and preparation of GrB-based fusion constructs. A, schematic diagram of GrB fusion constructs containing scFv 4D5 and GrB without or with fusogenic peptide 26. B, purified GrB-based chimeric proteins were analyzed by SDS–PAGE under nonreducing conditions.

Expression, purification, and activation of GrB-based proteins

The GrB-based proteins were expressed in HEK 293T host cells and purified by immobilized metal affinity chromatography as described in Supplementary Methods.

Determination of K_d by ELISA

The K_d value and specificity of GrB-based protein samples were evaluated by ELISA on Her2/neu extracellular domain (ECD), Her2/neu-positive BT474 M1 cells, and Her2/neu–negative Me180 cells. Rabbit anti-c-myc antibody and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G were used as tracers in this assay, as described previously (24).

GrB activity assays

The enzymatic activity of the GrB component was determined in a continuous colorimetric assay using *N*-α-*t*-butoxycarbonyl-L-alanyl-L-alanyl-L-aspartyl-thiobenzylester (BAADT) as a specific substrate (18). Assays consisted of commercial human GrB (Enzyme Systems

Products) or GrB-based fusion proteins in BAADT at 25°C. The change in absorbance at 405 nm was measured on a Thermomax plate reader. Increases in sample absorbance were converted to enzymatic rates by using an extinction coefficient of 13,100 cm^{-1} $(mol/L)^{-1}$ at 405 nm. The specific activity of GrB-based fusion proteins was calculated using native GrB as the standard.

Internalization analysis

Immunofluorescence-based internalization studies were conducted using BT474 M1 and Me180 cells. Cells were treated with 25 nmol/L GrB/4D5/26 for 4 hours and subjected to immunofluorescent staining with anti-GrB antibody [fluorescein isothiocyanate (FITC)-conjugated secondary antibody]. Nuclei were counterstained with propidium iodide (PI). Visualization of immunofluorescence was conducted with a Zeiss LSM510 confocal laser scanning microscope Zeiss LSM510 (Carl Zeiss).

In vitro cytotoxicity assays

Log-phase cells were seeded ($\sim 5 \times 10^3$/well) in 96-well plates and allowed to attach overnight. Cells were further incubated with various concentrations of GrB-based fusion proteins, GrB, or medium at 37°C for 72 hours. Cell viability was determined using the crystal violet staining method as described previously (23).

Annexin V/PI staining

The Annexin V/PI staining assay was used to quantitatively determine the percentage of cells undergoing apoptosis after exposure to GrB/4D5/26. Cells were seeded onto 6-well plates (5×10^5 cells/ well) and incubated with 100 nmol/L GrB/4D5/26 at 37°C for 24 or 48 hours. Aliquots of cells were washed with PBS and then incubated with Annexin V–FITC antibody. PI solution was added at the end of the incubation, and the cells were analyzed immediately by flow cytometry.

Cytochrome c release assay and Bax translocation

After treatment with GrB/4D5 or GrB/4D5/26, cells were collected and resuspended with 0.5 mL of 1× cytosol extraction buffer mix (BioVision) and then homogenized in an ice-cold glass homogenizer. The homogenate was centrifuged, and the supernatant was collected and labeled as the cytosolic fraction. The pellet was resuspended in 0.1 mL of mitochondrial extraction buffer and saved as the mitochondrial fraction. Aliquots of each cytosolic and mitochondrial fraction were analyzed by Western blotting with antibodies recognizing cytochrome c and Bax (Santa Cruz Biotechnology).

Assays for caspase activation and apoptosis

Western blot analysis was used to identify activation of caspases-3 and -9 as well as PARP cleavage. In addition, apoptosis was analyzed using antibodies recognizing Bcl-2 and BID (Santa Cruz Biotechnology).

Impact on cell signaling pathways

After treatment, cell lysates were analyzed by Western blotting with antibodies recognizing Her2/neu and phosphorylated (p)-mTOR (S2448; Cell Signaling Technology) as well as p-Her2/neu (Tyr877), p-Her2/neu (Tyr 1221/1222), EGF receptor, p-EGF receptor (Thr845), Her3, p-Her3 (Tyr1328), IGF1 receptor, p-IGF1 receptor (Tyr 1165/1166), estrogen receptor (ER), progesterone receptor (PR), Akt, p-Akt, extracellular signal–regulated kinase (ERK), p-ERK (Thr 177/Thr 160), PTEN, proteinase inhibitor 9 (PI-9), and β-actin (all from Santa Cruz Biotechonology). Immunoreactive proteins were visualized by enhanced chemiluminescence.

In vivo efficacy studies

We used Balb/c nude mice to evaluate the *in vivo* effect of GrB/4D5/26 against aggressive breast cancer. Each mouse received a weekly subcutaneous injection of 3 mg/kg estradiol cypoinate (26) starting 2 weeks before the injection of 1×10^7 BT474 M1 cells into the right flank. On the third day after cell inoculation, mice were injected intravenously (tail vein) either with saline or GrB/4D5/26 (44 mg/kg) 5 times per week for 2 weeks. Animals were monitored and tumors were measured (calipers) for an additional 50 days.

Immunofluorescence analysis

Twenty-four hours after the final injection of saline or GrB/4D5/26, mice were sacrificed and tumor samples were frozen immediately in preparation for section slides. The sample slides were incubated with either anti-GrB antibody (FITC-conjugated secondary antibody) or a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction mixture, as well as with an anti-mouse CD31 antibody (phycoerythrin-conjugated secondary antibody), and were further subjected to nuclear counterstaining with Hoechst 33342. Immunofluorescence observation was conducted under a Zeiss Axioplan 2 imaging microscope (Carl Zeiss).

Results

Construction, expression, and purification of GrB-based fusions

The sequence of the humanized anti-Her2/neu scFv 4D5 was derived from the published Herceptin light- and heavy-chain variable domain sequences (27). Previous observations suggested that use of fusogenic peptides facilitates endosomal escape and delivery of large molecules into the cytotol (28, 29). We therefore incorporated the fusogenic peptide 26 (Fig. 1A). GrB-based fusions were generated by fusing GrB to 4D5 with (designated GrB/4D5/26) or without (designated GrB/4D5) the addition of pH-sensitive fusogenic peptide 26 (AALEALAEALEALAEALEALAEAAAA) to the C-terminal of the construct. Furthermore, GrB and GrB/26 were used as controls. All fusion proteins were expressed in human embryonic kidney cells (HEK

293T). Following purification, the final products migrated at the expected molecular weights, with a purity of more than 95% (Fig. 1B).

Analysis of binding affinity

The binding affinities (K_d values) of GrB/4D5/26 and GrB/4D5 were assessed by ELISA using purified Her2/neu ECD, Her2/neu–positive BT474 M1 human breast cancer cells, and Her2/neu–negative Me180 human cervical cancer cells. Both fusions specifically bound to Her2/neu ECD and BT474 M1 cells but not to Me180 cells (Fig. 2A). The apparent K_d values were determined by calculating the concentration of fusion constructs that produced half-maximal specific binding. GrB/4D5 and GrB/4D5/26 showed apparent K_d values of 0.329 and 0.469 nmol/L, respectively, to Her2/neu ECD and 0.383 and 0.655 nmol/L, respectively, to BT474 M1 cells. These results are in general agreement with the published K_d value for native Herceptin to the Her2/neu receptor (0.15 nmol/L; ref. 27).

Enzymatic assay of GrB-based fusions

To assess the biologic activity of the GrB component of the fusions, we compared the ability of the constructs to cleave the substrate BAADT with that of native, authentic GrB (Fig. 2B). GrB/4D5 and GrB/4D5/26 had intact GrB enzymatic activity (1.54×10^5 and 1.57×10^5 U/μmoL, respectively). These activities were comparable with that of the native GrB standard (1.19×10^5 U/μmoL). Because the pro-GrB fusion constructs contain purification tags on the N-terminal end of GrB and render the molecule enzymatically inactive, these proteins were unable to cause hydrolysis of BAADT.

Cellular uptake and GrB delivery of fusion constructs

Immunofluorescence staining was conducted with BT474 M1 and Me180 cells. The GrB moiety of both fusions was observed primarily in the cytosol after treatment with a fusion protein in BT474 M1 cells but not in Me180 cells (Fig. 2C), showing that both constructs were efficient in cell binding and internalization after exposure to Her2/neu–positive cells. The internalization efficiency of the

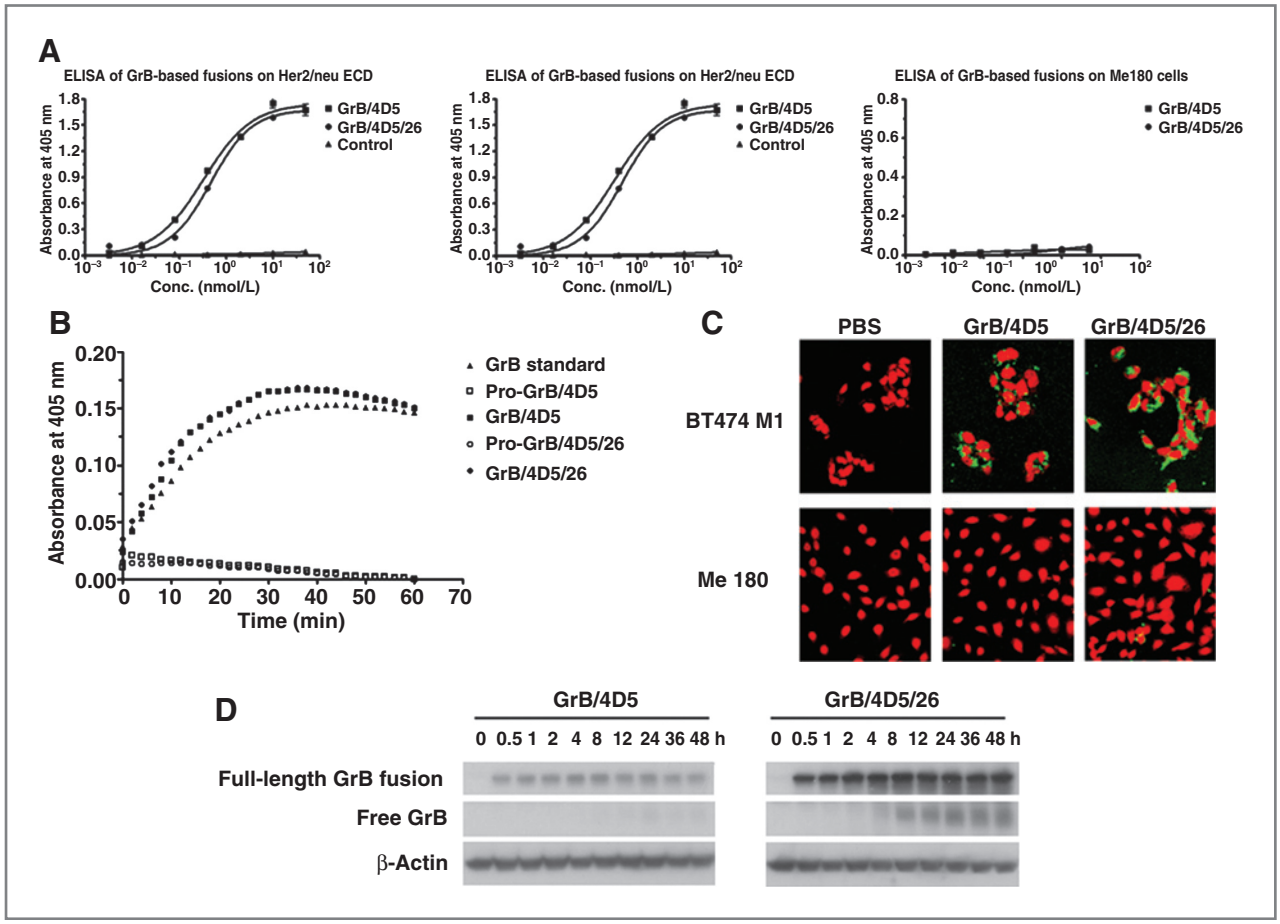


Figure 2. Characterization and comparison of GrB-based fusion proteins. A, K_d of the fusion constructs to Her2/neu ECD, Her2/neu–positive BT474 M1 cells, and Her2/neu–negative Me180 cells by ELISA. B, enzymatic activity of GrB moiety of fusion proteins compared with native GrB. C, internalization analysis of BT474 M1 cells and Me180 cells after 4 hours of treatment with 25 nmol/L functionalized GrB fusions. Cells were subjected to immunofluorescent staining with anti-GrB antibody (FITC-conjugated secondary), with PI nuclear counterstaining. D, Western blot analysis of intracellular behavior of 25 nmol/L GrB fusion constructs in BT474 M1 cells.

Table 1. Comparative IC_{50} values of GrB-based fusion constructs against various types of tumor cell lines

Cell line	Type	Her2/neu level	PI-9 level	IC_{50} (nmol/L) GrB/4D5/26	GrB/4D5	GrB/26	GrB
BT474 M1	Breast	++++	+	29.3	253.3	905.5	>1,500.0
Calu3	Lung	++++	+++++	40.5	242.4	863.0	>1,500.0
NCI-N87	Gastric	++++	+	90.4	629.0	1,106.0	>1,500.0
MDA MB453	Breast	+++	+	56.8	436.0	694.2	>1,500.0
eB-1	Breast	++	—	93.1	551.3	1,134.5	>1,500.0
MDA MB435	Breast	+	—	>500.0	>750.0	1,031	>1,500.0
Me180	Cervical	+	+	>500.0	>750.0	>1,500.0	>1,500.0

NOTE: +, indicates the Her2/neu expression level in different cancer cells.

fusions was further examined by time-dependent Western blot analysis of the GrB signal (full-length GrB fusion + free GrB; Fig. 2D). Both constructs internalized rapidly into BT474 M1 cells within 30 minutes. Compared with GrB/4D5, GrB/4D5/26 displayed enhanced and long-lasting cell internalization. The intracellular delivery of GrB after endocytosis of GrB/4D5 or GrB/4D5/26 also was assessed by time-dependent Western blotting (free GrB). We observed no GrB delivery by GrB/4D5 up to 48 hours of treatment, whereas GrB delivery by GrB/4D5/26 was observed starting at approximately 4 hours of treatment and presented a tremendously high level of free GrB up to 48 hours (Fig. 2D).

In vitro cytotoxic effects of GrB-based fusions

GrB-based fusions were then tested against a number of tumor cell lines. After 72 hours exposure, GrB/4D5/26 showed specific cytotoxicity to Her2/neu–positive cells, with IC_{50} values of less than 100 nmol/L (Table 1), and GrB/4D5 showed cytotoxic effects at somewhat higher doses (>200 nmol/L). In addition, GrB/26 showed minimal cytotoxicity at doses more than 600 nmol/L, but no significant activity of GrB itself was observed at doses up to 1.5 μmol/L. When Her2/neu–positive MDA MB453 cells were pretreated with Herceptin (5 μmol/L) for 6 hours and then treated with GrB/4D5/26 for 72 hours, the cytotoxicity of GrB/4D5/26 was reduced (Supplementary Fig. S1), thereby showing a requirement for antigen binding of the GrB/4D5/26 construct.

We further investigated the expression levels of the endogenous PI-9 in different tumor cells (Supplementary Fig. S2 and Table 1). These studies failed to find an association between the response of cells to the cytotoxicity of the GrB constructs and the endogenous expression of PI-9. This may suggest that factors other than PI-9 may account for the observed differences in GrB/4D5/26 cytotoxicity to Her2/neu–expressing target cells.

Cytotoxic effects of GrB/4D5/26 against cells resistant to Herceptin or lapatinib

Acquired resistance to Herceptin or lapatinib can be mediated by concomitant upregulation of Her2/neu downstream signaling pathways or activation of signaling through the ER pathway (30). In this study, we developed a model of Herceptin- and lapatinib-resistant variants of BT474 M1 cells. Parental BT474 M1 cells were readily sensitive to both Herceptin (IC_{50}, 52.5 nmol/L) and lapatinib (IC_{50}, 34.7 nmol/L; Table 2). Herceptin-resistant cells showed resistance to Herceptin [IC_{50}, 10.1 μmol/L; fold resistance (FR), 192] but remained sensitive to lapatinib (IC_{50}, 32.4 nmol/L). Lapatinib-resistant cells showed resistance to high micromolar concentrations of both Herceptin (IC_{50}, 74.1 μmol/L; FR, 1411) and lapatinib (IC_{50}, 8.2 μmol/L; FR, 237). As shown in Table 2, cells

Table 2. Cytotoxic effects of Her2/neu–targeted therapeutic agents on IC_{50} values in BT474 M1 cells and resistant variants

Agent	IC_{50} (nmol/L) with (FR)[a] BT474 M1	BT474 M1 Herceptin-resistant	BT474 M1 lapatinib-resistant	BT474 M1 + EGF[b]	BT474 M1 + NRG-1[c]	BT474 M1 + β-estradiol[d]
Herceptin	52.5 (1)	10,100.5 (192)	74,100.0 (1,411)	26,305.0 (501)	23,033.0 (439)	74.1 (1)
Lapatinib	34.7 (1)	32.4 (1)	8,225.0 (237)	543.0 (16)	547.1 (16)	33.9 (1)
GrB/4D5/26	32.9 (1)	26.8 (1)	66.1 (2)	21.7 (1)	18.1 (1)	31.3 (1)

[a]FR represents IC_{50} of agent on BT474 M1–resistant variants/that on BT474 M1 parental cells.
[b,c,d]Cells were pretreated with [b]20 ng/mL EGF, [c]50 ng/mL NRG-1, or [d]10 ng/mL β-estradiol for 72 hours before drug treatment.

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